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# RELATION BETWEEN BODY WEIGHT AND CAFFEINE TOXICITY IN *BUFO ARENARUM* HENSEL

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There is no uniform criterion for determination of the inter-relation existing between body weight and toxicity of drugs. In many instances it is found that in larger animals injected with a dose determined by unit of weight, the percentage of mortality or the severity of the intoxication is much higher than in smaller ones. For instance, Bahrena (1937) found that mortality with a given dose of strophanthin for the frog of 20 gm is 4%, and 88% for the frog of 50 gm. On the other hand Kisselt (1915) established that  $DL_{50}$  for caffeine injected intraperitoneally in rats is the same for animals under 40 gm and those over 120 gm.

It is generally agreed that standardization of dosage based on body weight is inaccurate. Clark (1937) maintains that it is impossible to formulate a general law based on this relationship. In a study of anaesthetic dosage of nembutal in cats and dogs Bazett and Erb (1933) found that the data could not be plotted as a straight line on either logarithmic or semi logarithmic paper. The curve was somewhat S-shaped but when the data were plotted on logarithmic probability paper a straight line could be obtained. They believe that from such curves the relationship of dosage to body weight can be predicted with considerable accuracy. However this method would require considerable modification to apply generally to different drugs and different species. Calculation of dosage based on drug surface area relationship has been proposed by Moore and by Dreyer and Walker. This has a closer connection with the volume of body water through which the drug circulates and consequently its distribution concentration in the body organs. Neither of these methods however, takes into consideration the body organ's metabolic processes especially those of detoxification.

We chose caffeine for our study because through its uniform distribution in nearly all the organs (Krupski, Kuns and Almásy 1934 Bock 1920) its dosage per body weight gives an approximate concentration proportional to the injected dose. Furthermore the elimination and detoxication take place practically within two or three hours (Eichler 1938) and mortality rates can be determined within this period. Surviving animals are normal the following day.

Having determined the  $DL_{50}$  we tried using this to find a mathematical formula which could allow us to adjust the dosage to animals of any weight.

**METHOD** Mean lethal dose was determined in *Bufo arenarum* Hensel, injecting a 10% solution of caffeine-sodium benzoate (Merck) in Ringer into the ventral lymph sac. This mixture contains 38% of pure caffeine. To obviate the influence on body weight of the ovaries the mass of which is variable only males were used.

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EXPERIMENTAL RESULTS A) *Determination of  $DL_{10}$* . The mean lethal dose found is shown in table 1. Mean lethal dose for females is 1 gm of caffeine sodium benzoate per kg (24°C). For males it is 0.9 gm. Fileline (1886) found that the mean lethal dose for caffeine is 0.1% of the body weight in the European frog.

B) *Symptomatology of the caffeine intoxication*. Following the injection of toxic doses of caffeine in toads, there can be seen a very marked hyperreflexia and a hypertonus of the muscles (fore legs in flexion, hind legs in extension), going from a slowing of all movements to complete fixedness. Death occurs

TABLE 1

*Determination of  $DL_{10}$  with a 10% solution of caffeine sodium benzoate in *Bufo arenarium* Hensel (female)*

Injection into the abdominal lymph sac Room temperature 24°C Time 17h (26 III 43) Ten toads injected with each dose Period of observation 24 hours

Dose of CAFFINE SODIUM BENZOATE (mg / kg)	600	700	800	900	1000	1100	1200	1300
Weight in grams	157	195	155*	115	130	115	170*	115*
	100	120	200*	105	135	100*	100*	105*
	90	75	100	90	125	80	115*	95*
	100	185	155*	170*	110	85	140*	115*
	120	160	185*	110	105	150	140*	85*
	130	175	120*	105*	105	115	100*	80*
	120	185*	125	160*	175*	100	80*	105*
	103	95	90	120	135*	105	100*	95*
	115	115	135	120	145*	95	85	95*
	105	70	160*	155*	75	80	85*	90*
Observed mortality	0/10	1/10	6/10	4/10	3/10	1/10	9/10	10/10
Calculated mortality (Behrens)		1/37	7/37	11/34	14/31	15/25	24/25	
Lethal dose	$DL_{10}$				$DL_{10}$			$DL_{100}$

\* Dead

generally within two hours and the animals which escape it, in spite of a marked hypertonus, progressively return to the normal state.

Care was taken to record room temperature in all experiments since it was later proved to be of paramount importance in caffeine intoxication. The quotient  $Q = \frac{DL_{100}}{DL_0} = 2.16$  speaks in favour of a well defined toxicity of this substance.

C) *Influence of weight on mortality*. The various groups injected with increasing doses of caffeine per kg clearly show a higher mortality among the animals of larger weight. Such a fact led us to study more deeply the cause of this finding. First, it was necessary to establish if the above mentioned difference in mortality was significant or not. For that, two series were used, one of

large animals and the other of small ones. They were injected with doses of 0.9 and 1 g/kg of caffeine. The results can be seen in table 2. Using the figures given here the percentage of mortality of the small animals calculated with the formula of van der Waerden (1936) was 34.5% and for the large ones was 76%. This difference is significant.<sup>2</sup>

D) Correction for weight differences. The second step was to find a way to obtain a homogeneous mortality for all weights. It was obtained (B. Günther) by changing the exponent  $n$  in the following formula  $D = KW$  in this formula  $D$  = injected dose  $K$  = constant which changes for each exponent and for each dose, and  $W$  = body weight. The variation of the exponent in the formula

TABLE 2

Influence of weight on the toxicity of a 10% solution of caffeine sodium benzoate in the *Bufo arenarum* Hensel ♂ infected into the ventral lymph sac

Period of observation 24 hours

Room temperature	21°C.		22°C.		23°C.	
	11/IV/43	10/VII/43	11/VII/43	1.0 g./kg.	1.8 g./kg.	
Dose	Period of observation 24 hours					
Group	<90 g.	>113 g.	<90 g.	>113 g.	<79 g.	>113 g.
Weight in grams	78	142	85	130	78	113
	77	143	90	132	84	118
	60	182	83	137	83	127
	62	163	90	130	79	123
	73	149	80	145	69	118
	86	153	80	117	76	129
	78	158	90	127	66	114
	86	138	85	116		117
	81	160	83	126		116
	88	155	80	130		117
						126
Observed mortality	2/10	6/10	3/10	7/10	4/7	11/11

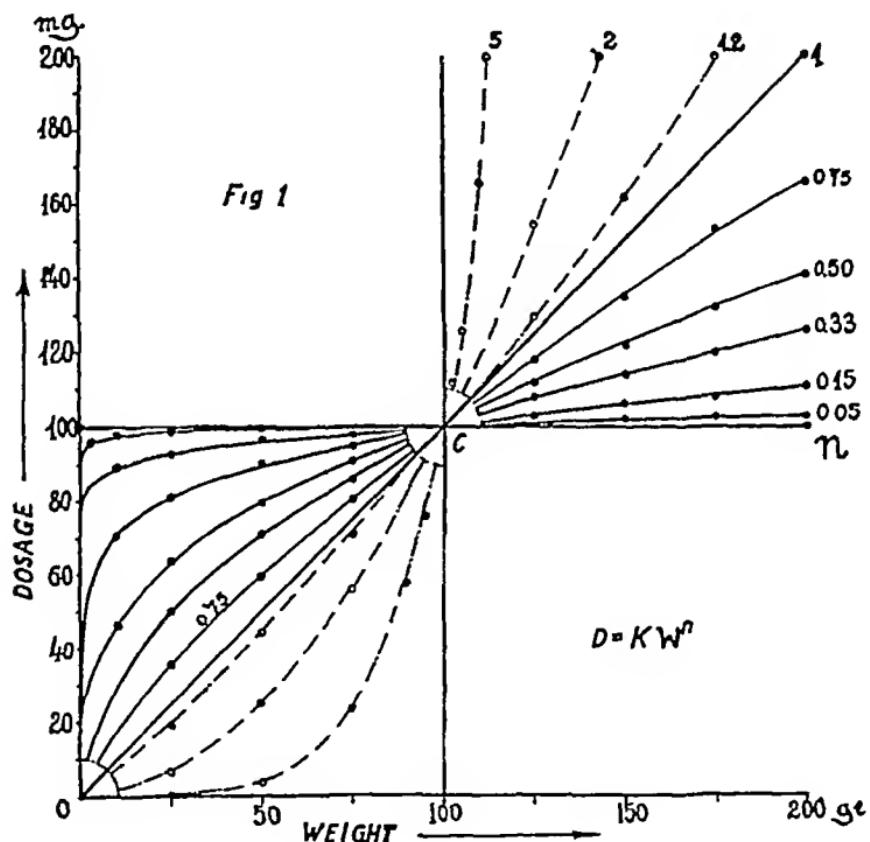
makes it possible to change the slope of dose curves in such a way that the mortality remains uniform as can be seen in the different curves of fig. 1. If with a given exponent the equalization of mortality cannot be attained some other must be tried (see fig. 1).

The mean lethal dose previously determined is taken as the reference dose or

$$^1 \text{The calculation is made in the following way } D = \sqrt{\frac{P_1 - P_2}{P_2 \cdot \frac{1}{n_1} + \frac{1}{n_2}}}$$

$D$  = difference which is significant when higher than 3.0  $P_1$  = percentage of mortality of the first group  $P_2$  = percentage of mortality of the second group  $p$  = percentage of total mortality;  $q = 100 - p$   $n_1$  = number of observations of the first group  $n_2$  = number of observations of the second group

some other one can be used, if desired. This dose is placed in the point of intersection of all the curves (point C, fig. 1). Starting from the general formula  $D = KW^n$ , we can determine  $K$  for a given exponent, because  $K = \frac{D}{W^n}$ . In such an equation  $D$  = mean lethal dose,  $W$  = mean weight of the animals injected when determining the aforesaid dose,  $n$  = exponent we want to use. Table 3 describes the manner in which the scale of doses is obtained, in the center of the system is the value of  $C = 100$  mg of caffeine sodium benzoate for 100 gm of frog's weight.



In table 3 there has been established the dosage for a number of weights, changing the exponent of the above mentioned formula. It is convenient to make a special table for each exponent in function of the weight. If it is needed to use other reference doses, the new value of the corresponding  $K$  must be calculated.

E) *Comparative mortality with the different exponents.* In table 4 various exponents have been applied and the mortalities in the different groups are compared. As we are dealing with a smaller number of animals, percentage of mortality was calculated with van der Waerden's formula (1936). The final

results of this series of experiments are represented in fig. 2. The results obtained with two reference doses, 0.9 and 1.0 g /kg are given

If caffeine is injected in direct relation with weight, i.e. with the exponent  $n = 1.0$ , it can be seen that the mortality of the large animals is far higher than that of the small ones with both reference doses. If the exponent 0.15 is used, i.e. the injections are made following the logarithm of weight, the percentage of mortality is inverted and deaths are more numerous among the small than among the large frogs

With 0.5 as exponent, mortality is nearly equal in large and small animals, but if we use a dose of 0.9 g /kg it can be seen that mortality with this exponent is higher among the small animals, that is the dose is too large for them. Using 0.66 this difference diminishes. The best results are obtained with the exponent 0.75. This value cannot be more accurately determined because the individual variations of susceptibility are especially marked in these animals

TABLE 3

Calculation of the different doses of caffeine sodium benzoate for the *Bufo arenarum* Hensel of Reference dose  $DL_{50} = 1 \text{ g /kg}$

$D = K \cdot W^n$	POWERS OF $W$ WITH DIFFERENT EXPONENTS $n$						PRODUCT OF THE CONSTANT $K$ , MULTIPLIED BY THE POTENCY $W^n$					
	1.0	0.75	0.66	0.50	0.33	0.25	1.0	0.75	0.66	0.50	0.33	0.25
Exponent =	1.0	0.75	0.66	0.50	0.33	0.25	1.0	0.75	0.66	0.50	0.33	0.25
Constant $K$	—	—	—	—	—	—	1.0	3.17	4.63	10.0	21.6	79.3
Weight $W$ in grams												
50	50	18.7	13.5	7.1	3.68	1.22	50.0	59.3	62.7	71.0	79.5	96.5
100	100	31.6	21.5	10.0	4.63	1.26	100.0	100.0	100.0	100.0	100.0	100.0
150	150	42.6	28.2	12.2	5.28	1.29	150.0	135.0	131.0	122.0	114.0	101.5
200	200	52.6	33.0	14.1	5.84	1.30	200.0	166.0	157.5	141.0	128.0	103.0

F) *Influence of metabolic intensity on mortality.* The above mentioned experiments show clearly that the small animals have better chances of resistance than the large ones. This could be explained by two mechanisms. 1) the small animal has, per unit of weight a higher amount of tissues that have an active part in the catabolism of the drug (liver kidney skin intestine) 2) with an equal amount of parenchyma metabolism might be higher per unit of weight among the small animals

With respect to the first possibility, Blank (1934) was able to prove in the frog and in the toad that the small animal has per unit of weight a greater amount of hepatic and renal parenchyma. Günther and de Soldati (1943) have confirmed this for the kidney in *Bufo arenarum* Hensel. This difference between small and large animals applies generally as has been shown by studies on weights of various organs in the same and in different species (Richet, 1895 Donaldson 1924 Clark, 1927 Kestner 1934 Kleiber and Cole 1937). With respect to the possible metabolic difference, there are data of Terroine and Delpech (1931) Liang (1934) and Blank (1934) Galli Mainini (1943 personal

communication) They all found constantly an enormous difference between the metabolism (oxygen consumption) of large and small frogs

TABLE 4

Comparison of mortalities applying different values in the formula  $D = K W^n$  to exclude the influence of weight on mortality with toxic doses of caffeine sodium benzoate in *Bufo arenarum* Hensel ♂

DATE	2/IV/43			3/IV/43			6/IV/43			7/IV/43			11/V/43		
	22			22			22.5			22			18		
Temperature C.	50 W <sup>0.22</sup>			10 W <sup>0.22</sup>			8 W <sup>0.22</sup>			4.2 W <sup>0.44</sup>			3.17 W <sup>0.22</sup>		
	S*	L	S	M	L	S	M	L	S	M	L	S	M	L	
Weight in grams	59	103	67	91	120	85	104	160	49	100	143	76	102	119	
	59	133	66	96	147	54	100	160	66	90	145	60	98	140	
	60	114	57	86	125	85	105	150	70	89	136	69	102	120	
	59	125	74	97	116	78	105	168	45	89	128	65	103	120	
	64	123	63	97	110	68	107	146	45	90	142	76	104	124	
	58	113	70	95	138	74	100	157	55	65	128	52	101	127	
	76	110	55	100	116	80	106	146	73	95	130	76	100	125	
	59	114	70	95	110	79	108	144	65	89	125	81	99	140	
		65	101	140	75	100	144	60	89	132	70	99	147		
		60	125	77	106	167	49	96	127	55	99	132			
		65			120							65	105	146	
		70			120							75	97	128	
		68			135							69	103	132	
		68			125							71	100	135	
		72			145							64	98	135	
		67			145										
		75			125										
		65			130										
		55													
		71													
Mortality observed after 48 hours	6/8	2/8	15/20	6/9	14/18	5/10	2/10	0/10	2/10	3/10	1/10	15/15	14/14	15/15	
% of mortality calculated according to v d Waerden	70	30	73	63	75	50	25	8	25	33	17	94	89	94	

$$p = \frac{Z+1}{n+2} \cdot 100\%, Z = \text{no of deaths}, n = \text{no of observations}$$

\* S = small, M = medium, L = large

The relation between temperature and metabolism has been described by Benedict (1932), Krogh (1941) and others. Increasing the general metabolism of the large animals by exposing them to a higher temperature should equalize this difference in metabolism.

Trying to verify this assumption, some groups of large toads (larger than 137 gm.) were placed in individual containers with water at 36°C and were kept in a thermostat at this temperature. Three hours later they were injected with

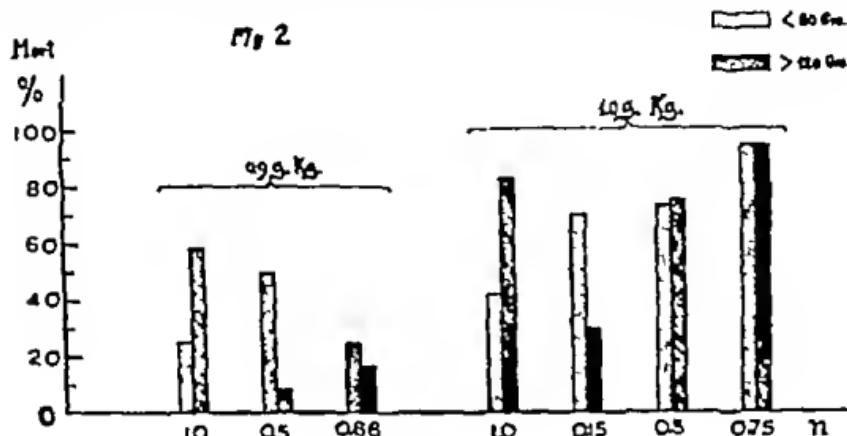


TABLE 5

*Influence of temperature on mortality with caffeine*  
Injection of 1 g./kg. of caffeine sodium benzoate into the ventral lymph sac of  
*Bufo arenarum* Hensel ♂

Group	L <sub>1</sub>	L <sub>2</sub>	S
Temperature °C.	34	15	16
Weight (in grams)	147 137 147 140  147 145 157 140  143 140 145 149	142 149 142 150  135 151 153 140	75 70 82 70  84 51 77
Observed mortality in 24 hours	5/8	12/12	7/7

a dose of 1 g./kg. of caffeine sodium benzoate and kept in the thermostat till the following day

Some groups of large and others of small animals (see table 5) were injected with the same dose simultaneously and they were left at room temperature

(16°C) All of them showed a pronounced hypertonus of all extremities and died without exception

Among the large animals placed at 36°C some presented a marked hypertonus and died. Those surviving were quite normal the following day. At room temperature of 16°C, the dose of caffeine used is  $DL_{100}$  even for the small animals, but among the eight large animals left at 36°C, three survived. This proves clearly the protecting action of the metabolic increase caused by the thermic elevation

With the dose of 1 g/kg injected into normal toads we found that with a temperature of 16 to 18°C the mortality was 23/23 (including the results of other series), but at 36°C, mortality was 3/8, a difference which is significant

**DISCUSSION** Among homeothermals as well as among poikilothermals, it has been shown that the small animals have per unit of weight a higher percentage of visceral parenchyma and a more intense metabolism than the large ones. For the mammalian, Kleiber (1932) and Brody (1932) established that the basal metabolism in function of the weight can be expressed in the following way  $Q = 70.5 W^{0.75}$

It is interesting to point out the analogy existing between this general metabolic formula and the parabolic function for the detoxication of caffeine in the toad ( $DL_{50} = 3.17 W^{0.75}$ ). In the poikilothermals, as well as in the homeothermals, the basal metabolism ( $W^{0.75}$ ) is the result of the sum of all the metabolic processes within the tissues, especially of the muscular ( $W^{1.0}$ ) and visceral ( $W^{0.5}$ ) metabolism (Günther, 1944)

Our experiments would point towards the whole metabolism and not that of special organs taking part in the process of caffeine detoxication

Dreyer and Walker (1914) analyzed the experimental results of numerous investigators, with reference to the toxicity of substances of quite different composition and origin (diphtheria toxin, arsenic acid, codeine hydrochloride, morphine, physostigmine sulphate, atropine sulphate, poisons of snakes, adrenaline, tetanus toxin, potassium chloride and caffeine) tried in various mammals. They arrived at the conclusion that it is more exact to relate toxicity to body surface. The exponent which they prefer is 0.72, that is, the same as we found for the *Bufo arenarum* Hensel intoxicated with caffeine. With this, the above mentioned authors have shown the general application of this exponent, which they believe is directly related to body surface (hypothesis first proposed by Moore, 1909) and to the total volume of blood. This conclusion is questionable because there are many organs and functions included in the metabolic formula mentioned above and any one of them could easily effect the result

On the other hand, a special case is the determination of the minimal lethal dose of the digitalis bodies (per kg /body weight), which is the same for young and adult cats (Haag and Corbell, 1940). The most exact relation was found by Chen, Bliss and Robbins (1942) on giving digitalis to cats according to the following formula, dose to  $2/3$  power of fresh cardiac weight. They interpret this formula as a relation with the size or surface of the heart, but really it must be a function that represents cardiac metabolism. If the metabolism is ex-

pressed per unit heart-weight, it gives an exponent approximate to  $-0.33$ . The value of  $-0.33$  was found also to represent the metabolism of skeletal muscle and that of other tissues (Meyerhof and Hinrich 1924, Kleiber 1941). These relations suggest a parallelism between cardiac muscle metabolism and digitalis dosage.

From the above mentioned experiments it can be deduced that there is a close relationship between caffeine toxicity and the metabolic activity of all tissues

#### SUMMARY

- 1) With animals of 100 gm the  $DL_{50}$  for caffeine sodium benzoate is 1.0 g/kg in the female of *Bufo arenarum* Hensel, and 0.9 g/kg. for the male at  $24^{\circ}\text{C}$ .
- 2) If a given dose per unit weight is injected, mortality is higher with large animals than it is with the small ones.
- 3) This difference disappears when the drug is injected according to the following formula  $D = K W$ , obtaining then a mortality quite uniform, when the exponent  $n$  is 0.75.
- 4) The relatively smaller metabolism of the larger animals can be compensated by keeping them in a higher temperature.
- 5) There is a close relation between the intensity of metabolism and the processes of detoxication of caffeine.
- 6) The application of the procedure under study to other drugs and species is discussed.

It is a pleasure to express our appreciation to Professor B. A. Houssay for his interest in this work.

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# THE POSTERIOR PITUITARY LIKE ACTIVITY OF COMMERCIAL ANTERIOR PITUITARY PREPARATIONS

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It has recently been reported by Sayers, White and Long (1) and Chance and Middleton (2) that their laboratory preparations of one of the anterior pituitary principles contained considerable amounts of posterior pituitary like activities. Since certain anterior pituitary fractions were to be investigated for their effect on water and electrolyte metabolism in view of the above reports it was thought advisable to test the preparations for posterior pituitary like factors. These tests were extended to nine commercial anterior pituitary preparations available at the time. To make an identification with posterior pituitary principles as close as possible the antidiuretic and vasopressor as well as the oxytocic activity of the anterior pituitary preparations was estimated. The resistance to alkali of any posterior pituitary like activity found was also investigated.

**Methods** *Assay of antidiuretic activity* Intravenous injections into unanesthetized rabbits were employed. (Walker (3) Heller (4))

*Assay of pressor activity* Spinal cats were used. Injections of the test solution were interposed between injections of a standard dose of Pituitary (posterior lobe) Extract.

*Assay of oxytocic activity* Virgin guinea pigs were used.

*Investigation of the alkali resistance of posterior pituitary-like activities in anterior pituitary extracts* It is known that the posterior pituitary principles are inactivated in a strongly alkaline medium. Dudley's (5) test was therefore applied to the anterior pituitary preparation investigated. The test consists in mixing a volume of extract with an equal volume of 2N sodium hydroxide. The mixture is allowed to stand for 2 hours at room temperature and is then carefully neutralized with 2N hydrochloric acid. Such neutralized solutions suitably diluted were tested for their antidiuretic, vasopressor and oxytocic activity.

Posterior pituitary extract (British Drug Houses) was used as the standard preparation. pH determinations were done with the B.D.H. capillary.

**RESULTS.** The results of the evaluation of the antidiuretic, pressor and oxytocic activities of the various anterior pituitary preparations investigated are given under the subsequent headings. Extracts available in powder form were not dissolved in the solvents provided by the makers as it was found that some of the solvents produced an oxytocic effect by themselves. The hormone powder contained in one ampoule was therefore dissolved in 10 cc saline or Ringer's solution.

*A. Anterior Pituitary Extracts* 1 *Anterior Pituitary Extract (Parke Davis & Co)* Formerly known as Antuitrin. Ampoules containing 10 cc. Makers description 'An aqueous acetic extract derived from 11 G (17 gr) of fresh anterior lobe of the pituitary gland. No claim is made for gonadotrophic or

skeletal-growth-stimulating activity" Content of preservative not stated, pH of extract 4.7

Thirty-two experiments with four different batches of this preparation were performed. The following amounts of posterior pituitary-like activities were found per cc: 1) an antidiuretic activity equivalent to 0.02-0.04 unit, or 20 to 40 milliunits (mU) posterior pituitary extract (fig 1), 2) an oxytocic activity equivalent to about 100 mU posterior pituitary extract (fig 2), 3) a pressor activity equivalent to about 100 mU posterior pituitary extract (fig 3). The

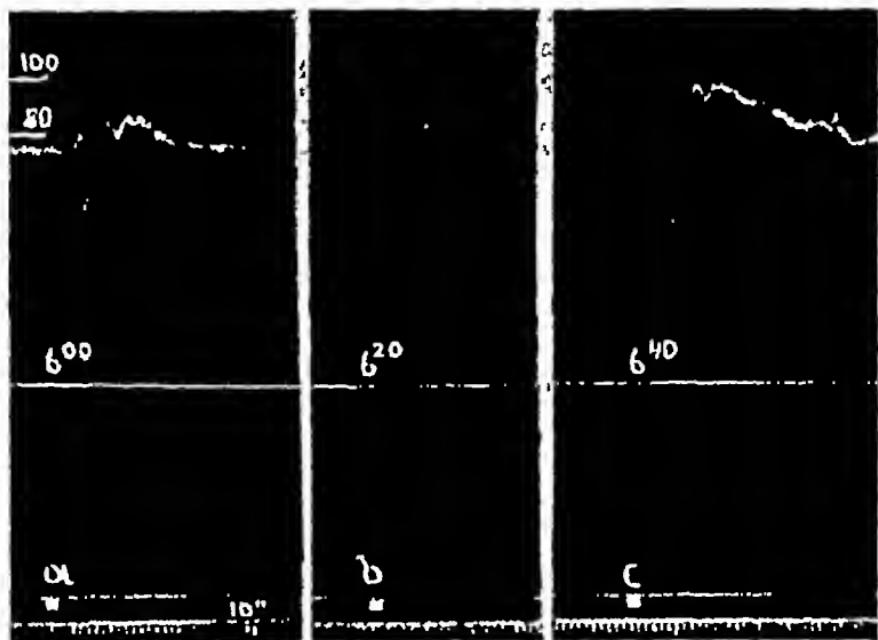


FIG 1 PRESSOR ACTIVITY OF ANTERIOR PITUITARY EXTRACT (PARKE, DAVIS & CO) BATCH III

Spinal cat. *a* = i.v. injection of 2.0 cc Anterior Pituitary Extract (dilution of original extract 1:8), treated with 2N sodium hydroxide. *b* = 20 mU posterior pituitary extract i.v. *c* = i.v. injection of 2.0 cc Anterior Pituitary Extract (Dilution 1:8) i.v. The results suggest that 2.0 cc Anterior Pituitary Extract diluted 1:8 i.v. containing 0.25 cc of the original extract contain the equivalent of more than 20 mU posterior pituitary extract i.e. the original Anterior Pituitary Extract appears to contain the equivalent of more than 80 mU posterior pituitary extract per cc. Note the fall of blood pressure preceding the pressor response of *a* and *c*.

pressor effects were regularly preceded by a depressor response. Most or all of the antidiuretic, oxytocic and pressor activity disappeared after treatment with 2N sodium hydroxide but the depressor response was not affected by the alkali. The fact that the oxytocic response disappeared while the depressor response persisted makes it unlikely that the unknown depressor substance was histamine. However, it may have been similar to the peptone preparation obtained by Hanke and Koessler (6) which according to these authors did not contract the guinea-pig uterus but lowered the blood pressure of the dog. The estimations

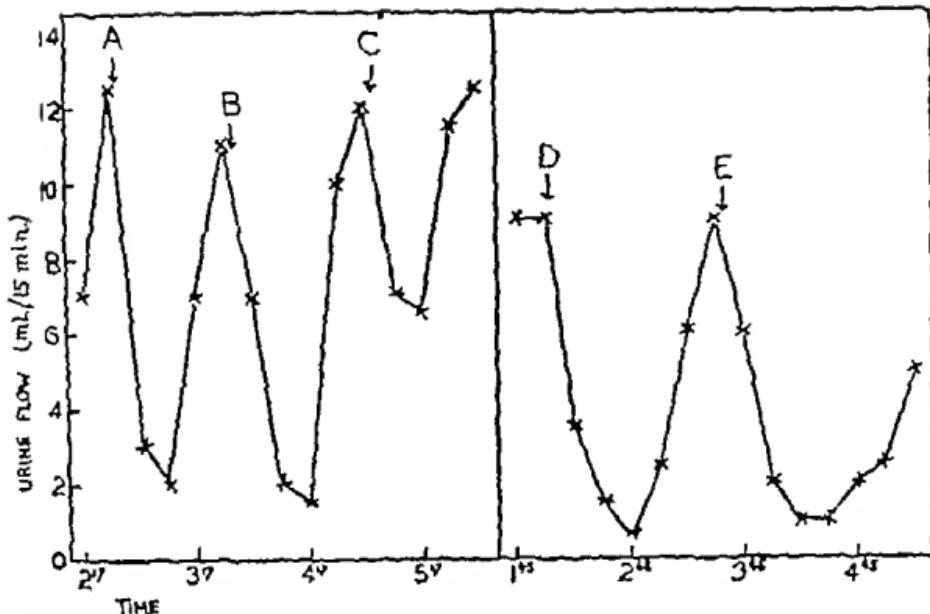


FIG 2 ANTIIDIURETIC ACTIVITY OF ANTERIOR PITUITARY EXTRACT (PARKE DAVIS & CO.)

I = rabbit no 17 At 10.32 a.m and 1.02 p.m 8% of body weight of water by stomach tube. A = 0.26 cc Anterior Pituitary Extract Batch II (Dilution 1/10) i.v. B = 1.0 mU posterior pituitary extract i.v. C = 0.6 cc Anterior Pituitary Extract (Dilution 1/10) treated with 2N sodium hydroxide i.v. II = rabbit No 12 At 10.30 a.m and 1.00 p.m water by stomach tube. D = 0.5 cc Anterior Pituitary Extract Batch III (1/10) i.v. E = 1 mU posterior pituitary extract i.v. It follows from A and B that batch II contained the equivalent of about 40 mU posterior pituitary extract per cc. C shows that most of the antidiuretic potency in the Anterior Pituitary Extract had been inactivated by the treatment with alkali. D and E suggest that batch III contained less antidiuretic activity than batch II.

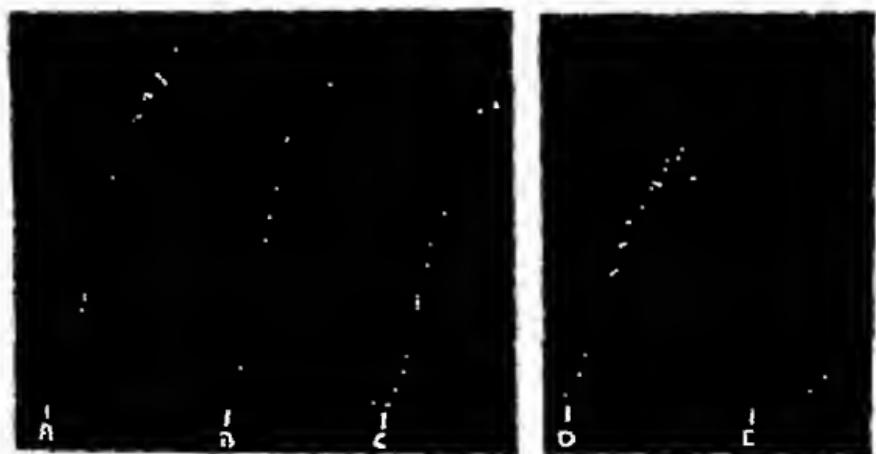


FIG 3 OXYTOCIC ACTIVITY OF ANTERIOR PITUITARY EXTRACT (PARKE DAVIS & CO.) ISOLATED UTERUS

A and C = 10 mU posterior pituitary extract. B = 0.7 cc Anterior Pituitary Extract, batch II (dilution 1/8). The uterus of a different animal was used for D and E. D = 2.5 mU posterior pituitary extract. E = 0.8 cc Anterior Pituitary Extract batch II (dilution 1/8) treated with 2N sodium hydroxide i.v. The oxytocic activity of batch II was apparently equivalent to about 100 mU posterior pituitary extract per cc.

of the pressor and the oxytocic activity agreed satisfactorily, but the possibility cannot be excluded that the discrepancy between these experiments and the estimations of the antidiuretic activity was due to the presence of the unknown depressor substance. The agreement between oxytocic and pressor estimations suggests that Anterior Pituitary Extract (Parke, Davis & Co) contained a posterior pituitary-like activity equivalent to approximately 100 mU/cc.

2 *Anterior Pituitary Extract* (Paines & Byrne, Ltd) Ampoules containing 1 1 cc. Makers' description "Equivalent to 30 grs of fresh substance" Content of preservative not stated, pH of extract 6.1

Seventeen experiments with two batches of this preparation were performed. The following amounts of posterior pituitary-like activities were found per cc. 1) an antidiuretic activity equivalent to 10 to 20 mU posterior pituitary extract, 2) an oxytocic activity equivalent to about 40 mU posterior pituitary extract, 3) a pressor activity equivalent to 20 to 40 mU posterior pituitary extract. The pressor effects obtained with one of the batches were preceded by a fall of blood pressure. All the posterior pituitary like activities disappeared after treatment with 2N sodium hydroxide but the depressor response persisted. The de pressor effect would, therefore, appear to have been due to a substance similar to the one contained in Anterior Pituitary Extract (Parke, Davis & Co).

The agreement between the results of the 3 tests and the inactivation by alkali suggest the presence of some posterior pituitary like substance in the extract. The amount present per cc would appear to have been equivalent to between 10 and 40 mU of posterior pituitary extract.

B *Gonadotrophic Fraction 1 Gonadotrophon* (Paines & Byrne, Ltd) Ampoules of powder. Makers' description "Gonadotropic hormone from 25 gr. fresh Anterior Pituitary" The Ampoules of Powder each contain 50 mU of hormone."

Thirty-five experiments with two batches of this preparation were performed. The following amounts of posterior pituitary-like activities were found per cc. 1) an antidiuretic activity equivalent to about 10 mU posterior pituitary extract, 2) a pressor activity equivalent to about 10 mU posterior pituitary extract, 3) the oxytocic activity of the first batch investigated equalled less than 20 mU posterior pituitary extract. The estimation of the oxytocic potency of the second batch met considerable difficulties in giving very variable results. Values for oxytocic activity ranging from the equivalent of 50 to the equivalent of more than 320 mU posterior pituitary extract were obtained. However, the oxytocic as well as the antidiuretic and pressor activities were found to have disappeared after treatment with 2N sodium hydroxide. The absence of a depressor effect suggests that the substance responsible for the unduly high oxytocic activity of Batch II was not a histamine-like factor. The inactivation of the oxytocic activity by alkali agrees with this assumption.

The high oxytocic activity of Batch II has not been considered in the final assessment of the average posterior pituitary like potency of this preparation.

2 *Synaploidin* (Parke, Davis & Co) Rubber-capped bottle containing 10 cc. of extract. Makers' description "A combination of chorionic gonadotrophin

(luteinizing hormone) from human pregnancy urine and the follicle-stimulating hormone (gonadal synergist) from the anterior lobe of the pituitary gland. Each cc contains 15 synergy rat units. Content of preservative 0.5% phenol pH of extract 6.8 to 7.0

Seventeen experiments with one batch of this preparation were performed. The following amounts of posterior pituitary-like activities were found per cc: 1) an antidiuretic activity equivalent to about 10 mU posterior pituitary extract, 2) an oxytocic activity equivalent to 10 to 40 mU posterior pituitary extract, 3) a pressor activity equivalent to about 10 mU posterior pituitary extract. The posterior pituitary-like activities disappeared after treatment with 2N sodium hydroxide. A depressor effect was observed in some instances but was only obtained after several samples of extract had been withdrawn from the bottle. This finding may suggest that it would be more advisable to keep the preparation in ampoules.

**C Growth Hormone 1 Anterior Growth (Parke Davis & Co)** Rubber capped bottle containing 20 cc. Makers description: An extract of the anterior lobe pituitary gland containing the growth hormone with small amounts of thyrotropic and gonadotropic hormones. Each cc contains 10 rat-growth units. Content of preservative 2.5% butanol pH of extract 6.6

Twenty two experiments with one batch of this preparation were performed. The following amounts of posterior pituitary-like activities were found per cc: 1) an antidiuretic activity equivalent to less than 4 mU posterior pituitary extract, 2) a pressor activity equivalent to less than 2.5 mU posterior pituitary extract, 3) an oxytocic activity equivalent to 40 to 60 mU posterior pituitary extract which disappeared completely after treatment with 2N sodium hydroxide.

The anterior pituitary growth hormone is extracted in an alkaline medium and the inactivation of any posterior pituitary activity may therefore be expected. The results of the tests for antidiuretic and pressor activity agree with this assumption. The oxytocic activity found was not due to a histamine-like factor (no depressor response inactivation by alkali).

**2 Arescon (Paines & Byrne Ltd)** Ampoules containing 1.1 cc. Makers description: Each 1.1 cc contains growth hormone from 2 gms of fresh anterior pituitary. Content of preservative not stated pH of extract 6.6 to 6.8

Fourteen experiments with two batches of this preparation were performed. No posterior pituitary-like activity was found.

**D Lactogenic Fraction 1 Physolactin (Glaxo Laboratories Ltd)** Rubber capped bottle containing 15 cc of extract. Makers description: 'Active lactogenic principles of the pituitary. Contains not less than 60 Riddle-Bates units of prolactin in each cc. Content of preservative 0.5% phenol pH of solution 8.0 to 8.2

Twenty-six experiments with three batches of this preparation were performed.

The lactogenic hormone or the anterior pituitary may be extracted in an acid or an alkaline medium. The present preparation was found to be strongly alkaline and contained no appreciable amount of posterior pituitary-like activity.

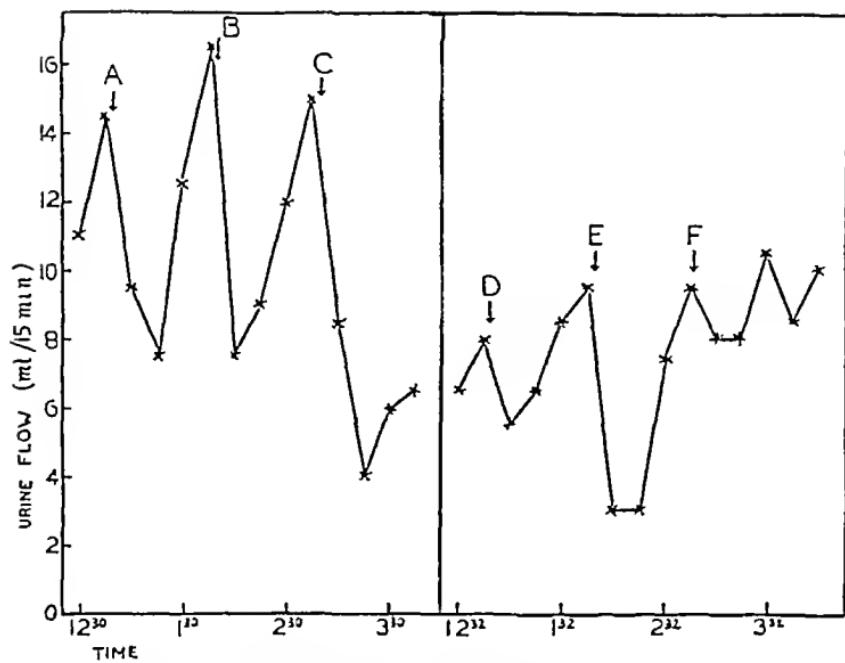


FIG. 4 ANTDIURETIC ACTIVITY OF THYROGAN BATCH II

I = rabbit No 15 At 10 10 and 11 54 water by stomach tube A = 0.25 cc Thyrogan (dilution 1 2500) x B = 1.0 mU posterior pituitary extract x C = 0.38 cc Thyrogan (dilution 1 2500) x II = rabbit No 19 At 10 12 and 11 56 water by stomach tube D = 1.0 mU posterior pituitary extract x E = 0.5 cc Thyrogan (dilution 1 2500) x F = 0.5 cc Thyrogan (dilution 1 2500) treated with 2 N sodium hydroxide x These results suggest an antidiuretic activity equivalent to more than 7500 mU (C) and to approximately 10 000 mU (B) posterior pituitary extract per cc It was completely inactivated by the treatment with alkali (F)

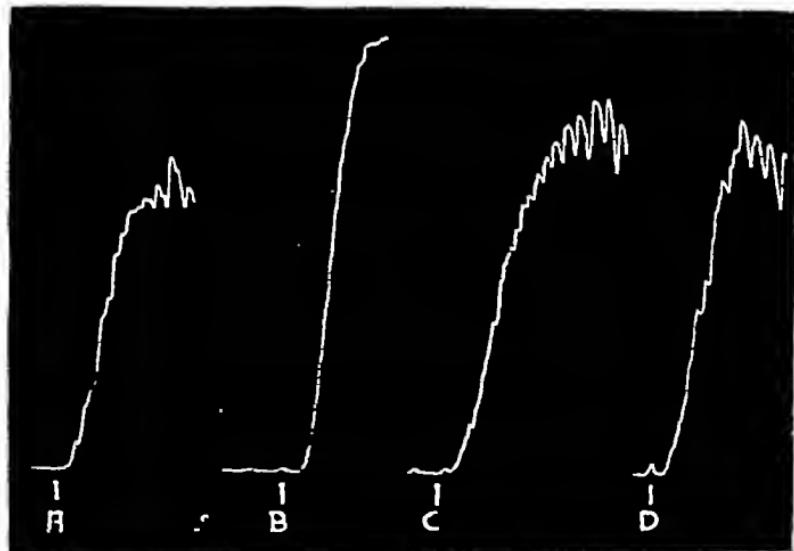


FIG. 5 OXYTOCIC ACTIVITY OF THYROGAN BATCH II ISOLATED I TERUS

A and C = 2.5 mU posterior pituitary extract B = 0.2 cc Thyrogan (dilution 1 800) D = 0.2 cc Thyrogan (dilution 1 1600) These results indicate an oxytocic activity equivalent to more than 10 000 mU posterior pituitary extract per cc

E Thyrotrophic Fraction 1 Thyrogen (The British Drug Houses Ltd)  
 Ampoules of powder Makers description 'Anterior pituitary thyrotropic  
 hormone Standardised biologically to contain 50 guinea pig weight units in

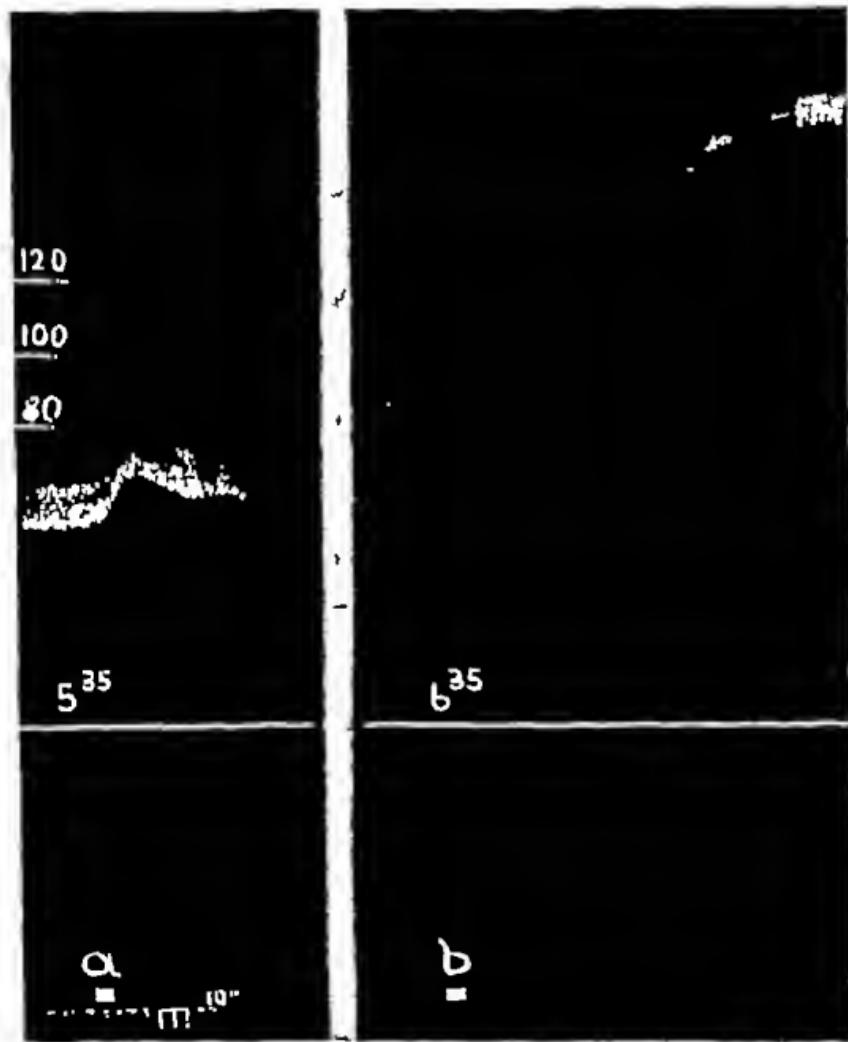


FIG. 6 PRESSOR ACTIVITY OF THYROGAN BATCH I

Spinal cat a = 50 mU posterior pituitary extract i.v. b = 0.5 cc Thyrogen (ondiluted)  
 The blood pressure response compares with the typical triphasic effect of large doses  
 of pituitary (posterior lobe) extract. The decrease of blood pressure after the preliminary  
 rise is usually interpreted as being due to coronary constriction

each ampoule derived from 2 to 2.5 gms of desiccated anterior pituitary substance. For intramuscular injection

Thirty experiments with four batches of this preparation were performed. The posterior pituitary like potency was found to vary considerably from batch

to batch. However, there was reasonable agreement between the results of estimations of the antidiuretic, pressor and oxytocic activities of any single batch. The determination of the posterior pituitary-like potency of the various batches gave the following results: Batch I, activity equivalent to between 1500 to 2000 mU/cc; Batch II, activity equivalent to between 8000 to 10,000 mU/cc; Batch III, activity equivalent to between 4000 to 8000 mU/cc; Batch IV, activity equivalent to less than 1000 mU/cc. It will be noted that the activity of batch II approached or equalled that of the Pituitary (posterior lobe) Extract B.P. (figs 4 and 5).

The bulk of the posterior pituitary-like activities was found to have disappeared after treatment with 2N sodium hydroxide. The antidiuretic, pressor and oxytocic effects observed were therefore likely to have been due to a posterior pituitary-like substance. This was even more strongly suggested by the effect of intravenous injections of larger amounts of the undiluted extract on the blood pressure of the spinal cat. Such injections gave the typical picture of the blood pressure effects of a large dose of pituitary (posterior lobe) extract (fig. 6). The preliminary rise, the subsequent fall (commonly ascribed to a transient constriction of the coronary arteries, Van Dyke (7)) and the secondary, more pronounced, rise of the blood pressure will be noted.

2 *Thyrotropin* (Pain's & Byrne, Ltd) Ampoules of powder. Makers' description "The ampoules of powder each contain 100 guinea pig units of hormone from approximately 1 gm fresh Anterior Pituitary."

The supply of this preparation was limited to 5 ampoules. The few (eight) experiments done suggested a negligible content of posterior pituitary-like factors.

**DISCUSSION** Complete separation of the posterior from the anterior pituitary lobe would seem to be difficult for anatomical reasons and the raw material for Anterior Lobe Extracts can therefore be expected to contain some posterior pituitary tissue. The chemical processes used for the purification and fractionisation of anterior pituitary extract eliminate the posterior pituitary activities in most cases, but recently published reports (1, 2), on laboratory preparations and the results of the present investigation suggest that this is not always the case and that occasionally posterior pituitary activities may even be concentrated.

The identification of the posterior pituitary principles in commercial anterior pituitary extracts is clearly a difficult undertaking as it cannot be excluded that the other substances present in such preparations will interfere with the estimation of posterior pituitary activity. This applies particularly to the estimation of oxytocic activity. It seemed advisable, therefore, to test for posterior pituitary-like potency by several methods. Autogenous and non-autogenous substances which stimulate the uterus or raise the blood pressure or inhibit a water diuresis are known. However, a preparation which exhibits all these three activities is likely to contain the posterior pituitary principles. A summary of the results of an investigation of nine commercial Anterior Pituitary extracts (or fractions of extracts) for posterior pituitary-like activity are given in table 1.

It should be pointed out that the amounts of posterior pituitary-like activity shown in table 1 have been found in 1.0 cc or, in the case of a dry extract, in one ampoule of the anterior pituitary preparation. Estimations of the anterior pituitary activities of these preparations were beyond the scope of this investigation. The relation of the concentrations of posterior pituitary-like to anterior pituitary activity can, therefore, not be stated.

In view of the agreements reached in Geneva in 1938 (8) and the progress in the purification of hormone fractions of the Anterior Pituitary Extracts made since, it seems likely that international standard preparations of Anterior Pituitary fractions will be elaborated in the near future and that such substances will be ultimately included in the pharmacopoeia. The laboratory reports mentioned and the results of the present investigation suggest that estimations of posterior pituitary-like activity should be included in the pharmacopoeial

TABLE 1

NAME OF PREPARATION	POSTERIOR PITUITARY-LIKE ACTIVITY PER CC. OF ORIGINAL PREPARATION (IN EQUIVALENTS OF MILLIUNITS OF PITUITARY (POSTERIOR LOBE) EXTRACT)	DEPRESSOR EFFECT
Anterior Pituitary Extract (Parke Davis & Co.)	40 to 100	+
Anterior Pituitary Extract (Paines & Byrne Ltd.)	10 to 40	+
Gonadotrophin (Paines & Byrne Ltd.)	10 to 20	-
Synapoidin (Parke Davis & Co.)	About 10	-
Antaltrin Growth (Parke Davis & Co.)	Negligible	-
Kreacone (Paines & Byrne Ltd.)	Negligible	-
Physiolutin (Glaxo Laboratories Ltd.)	Negligible	-
Thyrogan (British Drug Houses Ltd.)	Up to 10,000	-
Thyrotropin (Paines & Byrne Ltd.)	10 to 15	-

"Test for Purity" unless perhaps the process of preparation laid down has been shown to eliminate the posterior pituitary principles.

## SUMMARY

1 Nine commercial preparations of Anterior Pituitary Extracts and their fractions were investigated for their posterior pituitary activity.

2 The oxytocic, pressor and antidiuretic activities of each preparation were tested. The resistance to 2N sodium hydroxide of any of the posterior pituitary like factors found was determined.

3 Seven out of the nine preparations were found to contain negligible amounts of posterior pituitary activity. One preparation contained per cc. the equivalent of 40 to 100 mU of Pituitary (posterior lobe) Extract. The posterior pituitary like activity of another extract varied considerably from batch to batch, amounting in one case to the equivalent of about 10 units of Pituitary (posterior lobe) Extract per cc.

We wish to thank the Colston Research Committee for grants which defrayed the cost of this investigation

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# THE EFFECT OF TWO DIOXANE DERIVATIVES 883 AND 933F ON NORMAL DOGS AND ON ANIMALS WITH NEUROGENIC AND RENAL HYPERTENSION<sup>1</sup>

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It has been shown in a previous communication that hypertension resulting from section of the splanchnic nerves in the dog is the result of excitation of the sympathetic nervous system (1). Accordingly substances which decrease the activity of this system should be effective in reducing the blood pressure of animals with neurogenic hypertension. A series of compounds which inhibit the structures innervated by adrenergic nerves have been described (2). Among these the derivatives of dioxane first synthesized and described by Fournéau (3) are notable since in small but still effective quantities their toxicity appears to be low (4).

The effect of two of these Fournéau compounds, 883 and 933F, both derivatives of dioxane on anesthetized normal dogs has been described by Fournéau and Bovet (3). These investigators noticed a fall in blood pressure following the intravenous injection of these compounds. Handovsky, on the other hand, reported that the injection of 883F into unanesthetized animals resulted in a slight rise in blood pressure (5). Bovet and Simon were unable to notice any effect of 883F on the blood pressure of the unanesthetized normotensive animal (4).

The action of these dioxanes on carotid sinus reflexes was studied by Vleeschhouwer who found that they weakened the pressor reflex elicited by occlusion of the common carotid artery and decreased the depressor reflex produced by stimulation of the sinus nerve (6). Heymans and Bouckaert observed that the injection of from 5 to 10 mgm of 933F into unanesthetized dogs with chronic neurogenic hypertension resulted in a decline in blood pressure which lasted for several minutes (7).

The present paper deals with an investigation of the hemodynamic alterations caused by these dioxane derivatives, 883 and 933F in normal dogs and in animals with chronic neurogenic hypertension. Thanks to the kindness of Dr. Harry Goldblatt, the effects of 883F on the blood pressure of animals with renal hypertension could be incorporated in this report.

**MATERIAL AND METHODS.** A total of fifty-five experiments was performed on twelve unanesthetized normotensive dogs and on six animals made hypertensive by sectioning of the moderator nerves (8). Blood pressure records were taken from the femoral artery with the Hamilton manometer. The cardiac output was determined by means of the Fick principle according to the technique described in a recent communication (1). The total peripheral resistance was calculated according to the formula 
$$\frac{\text{Mean pressure} \times 1332}{\text{Cardiac output/second}}$$
 (9).

<sup>1</sup> This work was supported by a grant from the Commonwealth Fund.

This formula expresses the loss of pressure head in the circulatory system in absolute units, one unit representing 1 dyne  $\text{cm}^{-2}/\text{sec}$ . As the total peripheral resistance expresses the resistance to flow in the entire vascular tree, its deviations from control levels permit no conclusions on the localization of those changes.

From 2.5 to 5 mgm per kilogram weight of the compounds was dissolved in 10 cc of normal saline and injected into a leg vein over a period of 1 minute. The infusion fluids were prepared by dissolving from 2.5 to 5 mgm per kilogram weight of the dioxanes in from 100 to 150 cc of normal saline. This dosage was selected since it had been used by Bovet and Simon (4) and by Fournau and Bovet (3), who found the toxicity within this range to be low. The rate of infusion into the leg vein varied from 2.5 to 4.5 cc per minute. In thirteen instances the drugs were given by mouth. In these cases, 200 mgm of the powdered compounds were enclosed in gelatin capsules and fed at half hour intervals, and the blood pressure and the heart rate were recorded at 30-minute intervals. The total oral dosage varied from 40 to 100 mgm per kilogram weight. This wide range was chosen in order to obtain possible information on the toxicity of the orally administered compounds.

The greatest difficulty in evaluating the effect of the Fournau drugs on dogs with experimental neurogenic hypertension resulted from the instability of the blood pressure of these animals (8). Consequently, three control experiments were performed, in which the action of the intravenous infusion of normal salt solution on the blood pressure of animals with neurogenic hypertension was studied. The depressor effect of adrenaline, which has been observed after the administration of Fournau compounds (3), was used in order to ascertain whether or not the dioxanes were responsible for the hypotension which followed their oral administration. Five cc of an epinephrine solution (1:50,000) were injected into the leg or ear vein after from 6 to 800 mgm of the dioxane had been injected. A fall in blood pressure following the injection of the adrenaline solution was considered as evidence of the activity of the dioxane derivatives.

**I. EXPERIMENTS WITH 933F** *The Effect of the Injection and Infusion of 933F on Normal Animals* In three experiments the effect of the intravenous injection of from 2.5 to 5 mgm per kilogram weight of 933F on the blood pressure of normotensive dogs was investigated. As seen in table 1, the injection resulted in a slight rise in blood pressure of from 5 to 30 mm Hg, occurring 2 to 3 minutes after the onset of the injection. In every instance the elevation of the blood pressure was accompanied by a marked increase in the heart rate of from 120 to 150 beats per minute. These experiments confirmed the results of Handovsky, who observed that the injection of 933F into unanesthetized dogs was followed by a rise in blood pressure (5).

933F was infused into normal dogs in six experiments. Table 2 demonstrates that the blood pressure remained unchanged both during the infusion and for 40 minutes following it. The pulse pressure rose slightly, with the exception of one experiment (#14, 6/13/44) in which it fell 28 mm Hg. The heart rate increased an average of 100 beats per minute during the infusion. Slight salivation was the only toxic symptom present during the infusion.

Hemodynamic alterations were followed in three experiments. As seen in table 2, the minute volume rose slightly in one instance, remaining constant in the rest of the experiments. As the systolic discharge fell, the cardiac output was maintained at control levels by the increase in the heart rate. The total peripheral resistance remained unchanged, indicating that no changes in the resistance to flow in the vascular tree had occurred.

*The Effect of the Oral Administration of 933F on Normotensive Animals* In three cases, 800 mgm. of 933F were fed to animals with normal blood pressure (table 3). After ingestion of from 600 to 800 mgm. of the drug, the blood pressure fell slightly, while the heart rate increased an average of 130 beats per minute. During this period the injection of epinephrine (1:50,000) produced a fall in blood pressure ranging from 40 to 60 mm. Hg. The tachycardia persisted over periods ranging from 1 to 3 hours. Toxic symptoms were not present in animals #18 and #18. Dog #14 vomited twice during the experimental period but showed no other signs of toxicity. The results obtained on the cardiac output during the infusion of 933F made it probable that the increase in the heart rate observed during the oral administration of 933F was an important factor in the maintenance of the cardiac output and the blood pressure.

TABLE 1  
*Effect of the injection of 933F into normal dogs*

DOG	DATE OF EXPERIMENT	AMOUNT OF DRUG INJECTED IN 10 CC. SALINE		BEFORE CONTROL PERIOD	AFTER INJECTION	
					At lowest blood pressure	At highest blood pressure
14	5/31/44	72.6 mgm. (5 mgm. per kilo)	Blood Pressure Heart Rate Systolic/Diastolic	145 mm. Hg 150 234/140	150 (3.5) 270 241/112	180 (2)* 150 200/135
				90 mm. Hg 90 200/93	120 (3)* 210 200/86	165 (7) 150 222/74
				120 mm. Hg 60 192/83	150 (2.5)* 210 228/89	140 (8) 120 250/74

Number of minutes after the administration of the drug.

*Results Obtained with the Injection and Infusion of 933F into Animals with Experimental Neurogenic Hypertension* The effect of a single injection of from 45 to 100 mgm. of 933F was recorded in five hypertensive animals. Table 4 demonstrates that within 1 to 2 minutes after the injection there was a rapid decline of the blood pressure amounting to an average of 60 mm. Hg. This hypotensive effect persisted for from 5 to 15 minutes. Simultaneously the heart rate increased an average of 110 beats per minute returning to its control value 10 to 40 minutes later. Toxic symptoms were not observed. The fall in blood pressure observed in these experiments differentiates the action of 933F in hypertensive animals from that recorded in normal dogs in which 933F failed to affect the blood pressure.

The infusion of 933F, performed in three cases (table 5) was responsible for a fall in the blood pressure and a rapid rise in the heart rate immediately following the onset of the infusion. This hypotensive period however lasted only from

TABLE 2  
The effect of the infusion of  $833F$  into normal dogs

NO. OF DOG	DATE OF EXPERIMENT	AMOUNT OF DRUG INFUSED (IN 150 CC. VOLUME)	RATE OF RATE OF INFUSION cc./min.	EFFECT OF INFUSION OF DRUG		MEAN BLOOD PRESSURE mm. Hg	HEART RATE b.p.m.	MINUTE VOL. TIME cc./min.	O <sub>2</sub> INTAKE		CO-OP- ERATIVE INDEX OF O <sub>2</sub> UTILI- ZATION	O <sub>2</sub> INTAKE cc./min.	EXTERNAL- VENOUS DIFERENCE cc./min.	EXTERNAL RESISTANCE dynes cm. <sup>-4</sup> sec.	
				BEFORE	AFTER				REL. %	REL. %					
14	6/13/44	156 mgm (2.6 mgm /kilo)	4.5	Before	145	120 (34)*	240	238/137							
13	6/10/44	188.6 mgm (2.5 mgm /kilo)	4.5	Before	140	140 (21)*	210	106/111							
14	6/21/44	156 mgm (2.6 mgm /kilo)	4.5	Before	140	138 (40)*	3,460	208/104	38.5	20.9	5.34	25.6	185	3,230	
				After			3,470	180	250/110	19.3	21.5	5.70	26.9	198	3,175
13	6/22/44	188.6 mgm (2.5 mgm /kilo)	4.5	Before	130	140 (23)*	3,550	90	207/116	39.6	22.9	3.43	16.0	122	2,020
				After			4,810	210	231/100	23.0	23.1	3.53	15.3	170	2,320
1	6/26/44	96.8 mgm (2.5 mgm /kilo)	4.5	Before	160	140 (32)*	3,065	90	250/125	34.0	22.6	4.18	18.5	128	3,010
				After			2,735	150	230/128	18.3	24.0	2.32	9.6	63	4,000

\* Number of minutes after start of the infusion

TABLE 3  
The effect of oral administration of 833F on normal dogs

DOG	DATE	AMOUNT DIGESTED 833F	TIME	HEART RATE	BLOOD PRESSURE mm. Hg
14	8/4/44	Control	1 40	108	155
		400 mgm	1 45		
		200 mgm	2 10	100	160
		200 mgm	2 40	160	160
			3 10	210	130
			4 30	112	130
13	8/4/44	Control	1 50	100	120
		400 mgm	1 55		
		200 mgm	2 25	84	120
		200 mgm	2 55	126	100
			3 25	168	120
			4 35	250	80
18	8/4/44	Control	2 00	92	140
		400 mgm	2 05		
		200 mgm	2 85	96	140
		200 mgm	3 05	120	118
			3 80	220	98
			4 45	180	130

TABLE 4  
Effect of the injection of 833F into hypertensive dogs

DOG	DATE OF EXPERIMENT	AMOUNT OF DRUG INJECTED IN 10 CC. SALINE		DURING CONTROL PERIOD	AFTER INJECTION	
					At lowest blood pressure	At highest blood pressure
2	5/29/44	180 mgm (5 mgm per kilo)	Blood Pressure	175 mm. Hg	126 (2)*	205 (7.5)
			Heart Rate	150	280	160
			Systolic/Diastolic	274/169	199/100	340/183
7	5/30/44	105 mgm. (5 mgm per kilo)	Blood Pressure	180 mm. Hg	85 (2)	210 (5.5)*
			Heart Rate	210	260	180
			Systolic/Diastolic	278/147	176/66	285/194
3	5/31/44	105 mgm (5 mgm per kilo)	Blood Pressure	190 mm. Hg	100 (1.5)	160 (15)
			Heart Rate	150	180	150
			Systolic/Diastolic	270/175	190/85	206/180
2	6/13/44	45 mgm (2.5 mgm per kilo)	Blood Pressure	185 mm. Hg	124 (1)	220 (6)
			Heart Rate	180	270	240
			Systolic/Diastolic	278/143	212/98	320/180
7	6/24/44	52.5 mgm (2.5 mgm per kilo)	Blood Pressure	185 mm. Hg	110 (1)*	190 (30)
			Heart Rate	130	240	240
			Systolic/Diastolic	255/125	149/84	240/100

Number of minutes after the administration of the drug

2 to 6 minutes, and was succeeded by a hypertensive phase of from 10 to 20 minutes' duration. During this period the blood pressure rose and the heart rate remained elevated. This second phase was succeeded by a third one, occurring from 30 to 40 minutes following the onset of the infusion, during which the blood pressure and the heart rate gradually declined. The hypotension persisted for an average of 40 minutes before returning to its control level. The toxic symptoms observed during the infusion were not pronounced, they consisted in a moderate degree of salivation and a slight increase in the respiratory rate.

The hemodynamic changes occurring during this prolonged state of hypotension characterizing the third stage, are illustrated in table 5. Simultaneously with the fall in blood pressure and the slowing of the heart rate, there occurred a decrease in the pulse pressure, in the minute volume, and in the systolic discharge, while the difference in the oxygen content between the arterial and the mixed venous blood increased. The total peripheral resistance declined in every instance. These results contrasted with those observed during the infusion of 933F into normal dogs, in which the blood pressure remained constant, and the heart rate rose. It was apparent from these experiments that the fall in blood pressure produced by the infusion of 933F into animals with neurogenic hypertension was the result of a decline in the cardiac output, combined with a fall in the total peripheral resistance.

*Observations on the Oral Administration of 933F in Animals with Neurogenic Hypertension.* 933F was administered orally to three hypertensive dogs in doses ranging from 1 to 1.2 gm. (table 6). After from 0.6 to 0.8 gm. of the drug had been given, the blood pressure decreased, reaching normotensive levels in from 1 to 2 hours. During this period, the heart rate declined an average of 40 beats per minute. The blood pressure as well as the heart rate returned to their control levels in from 2 to 3 hours after the last dose of 933F had been administered. It was characteristic of this hypotensive phase that fluctuations of the blood pressure, which were usually encountered in animals with neurogenic hypertension, ceased, and the blood pressure became stabilized. Epinephrine injections (1:50,000) resulted in a decline in blood pressure of from 40 to 60 mm. Hg. Toxic signs observed during the oral administration of 933F consisted in a moderate degree of salivation, which was observed in two animals. In addition, these two animals appeared to be listless and apathetic. As the drug was given for a period ranging from 2 to 4 hours only, no information concerning its effect on prolonged administration was available. The results obtained with both oral and intravenous administration of 933F demonstrated, therefore, that this compound effectively lowered the blood pressure of animals with chronic neurogenic hypertension.

**II. EXPERIMENTS WITH 883F. The Effect of the Injection and Infusion of 883F on Normotensive Dogs.** The effect of the intravenous injection of 883F on the blood pressure and the heart rate of normal animals was studied in six experiments. Table 7 demonstrates that from 2 to 5 minutes following the onset of the injection, the blood pressure fell an average of 15 mm. Hg. Simultaneously,

TABLE 6  
The effect of the infusion of *GSIP* into hypertensive dogs

NO.	DATE OF EXPERIMENT	AMOUNT OF SALT INFUSED (IN 150 CC. BALLOON)	RATE OF INFUSION cc./min.	EFFECT OF INFUSION OF SALT	MEAN BLOOD PRESSURES mm. Hg	HEART RATE beats/ min.	SYSTOLIC/ DIASTOLIC	SYSTOLIC KCAL/ON CHARGE	AFTER KCAL ON CHARGE	O <sub>2</sub> ARTERIAL- VENOUS DIFERENCE	CO-OP- ERATIVE O <sub>2</sub> UPTAKE RELATION	O <sub>2</sub> TOLER- ANCE	PULMO- NARY HYPERTEN- SION dynes/cm. <sup>2</sup>
12	6/1/44	301 mgm. (6 mgm. /Hllo)	4.5	Before During During After	200 120 (6.5) 210 (10) 85 (42.5)	240 270 280 150	306/167 110/78	22.4 17.0 27.4	20.3 24.1 26.6	4.0 8.90 4.62	15.2 36.8 17.4	215 235 190	2960
3	6/12/44	128 mgm. (2.5 mgm. /Hllo)	4.5	Before During During After	190 110 (4)* 210 (10) 82 (90)*	4 110 190 200 140	335/211 100/78	27.4 18.6	22.5	5.64	24.7	170	2135
17	6/20/44	248 mgm. (2.5 mgm. /Hllo)	4.5	Before During During After	175 110 (3) 200 (12) 95 (50)*	6 000 190 210 120	223/164 100/78 114/74	33.3 23.4	4.36	18.7	263	2890	
												31.3	1245

Number of minutes after the administration of the drug.

the heart rate rose from 100 to 150 per cent of its control value. Toxic symptoms were absent during and following the injection.

The influence of the infusion of 883F on normotensive dogs was studied in four experiments (table 8). In every instance, a slight decline in blood pressure was noticeable, the effect persisting for a period of from 29 to 60 minutes. The

TABLE 8  
*The effect of oral administration of 883F on hypertensive dogs*

DOG	DATE	AMOUNT INGESTED 913F	TIME	HEART RATE	BLOOD PRESSURE	
					mm Hg	
2	7/28/44	Control	10 30	180	180	
		200 mgm	10 40			
		200 mgm	11 10			
		200 mgm	12 10	172	145	
		200 mgm	12 30			
		200 mgm	1 05	180	135	
		200 mgm	2 40	128	125	
			3 10	168		
			5 10	190	175	
3	7/28/44	Control	10 50	184	200-160	
		200 mgm	10 55			
		200 mgm	11 22			
		200 mgm	12 30			
			1 00	150	190-170	
		200 mgm	1 05			
		200 mgm	2 10	180	130-140	
			3 00	122	115	
			4 55	130	160-145	
			8 30	120	130-180	
		Next day				
			11 00	188	200-160	
3	7/20/44	Control	10 25	240	200	
		200 mgm	10 30			
		200 mgm	11 00			
			11 25	180	180	
		200 mgm	12 45		160	
			2 45	140	155	

heart rate rose from 60 to 150 beats per minute. All four animals showed increased salivation toward the end of the infusion.

Changes in cardiac output, systolic discharge, and the total peripheral resistance were followed in three instances. Table 8 demonstrates that, together with the fall in blood pressure, the systolic discharge fell, to more than 50 per cent of its control value. As the heart rate increased an average of 110 beats per minute, however, the minute volume fell only slightly (table 8). The total peripheral resistance did not change in two experiments, but declined in a third.

These experiments revealed that the hemodynamic alterations caused by the infusion of 883F into normal animals were similar to those observed during the infusion of 933F. In both cases the systolic discharge declined but the minute volume was maintained by an increase in the heart rate.

*The Effect of the Oral Administration of 883F in Normal Dogs.* Six hundred mgm of 883F were administered orally to three normotensive animals over a period of from 30 to 50 minutes (table 9). In each experiment the changes were similar to those observed during the infusion of that compound. Simultane-

TABLE 7  
*Effect of injection of 883F into normal dogs*

NO.	DATE OF EXPERIMENT	AMOUNT OF DRUG INJECTED		PREINJECTION CONTROL PERIOD	AFTER INJECTION	
					At lowest blood pressure	At highest blood pressure
10	2/15/44	55 mgm in 10 cc saline	Blood Pressure	105	94 (3)*	100 (5)*
			Heart Rate	79	143	142
			Systolic/Diastolic	178/84	178/83	178/80
4	2/15/44	70 mgm. in 10 cc saline	Blood Pressure	110	81 (3)*	
			Heart Rate	120	165	
			Systolic/Diastolic	162/90	141/80	
12	2/21/44	67.8 mgm. in 10 cc saline	Blood Pressure	125	106 (1.8)	125 (8)
			Heart Rate	110	220	140
			Systolic/Diastolic	204/125	148/92	204/125
6	2/23/44	105 mgm. in 10 cc saline	Blood Pressure	87	56 (3)	61 (8)*
			Heart Rate	185	240	240
			Systolic/Diastolic	163/75	88/44	122/51
9	3/7/44	60 mgm in 10 cc saline	Blood Pressure	122	106 (2)	140 (20)*
			Heart Rate	170	290	300
			Systolic/Diastolic	187/100	178/89	210/110
8	3/20/44	45 mgm in 10 cc. saline	Blood Pressure	124	110 (5)	125 (20)
			Heart Rate	120	240	190
			Systolic/Diastolic	223/118	212/112	223/105

\* Number of minutes after administration of the drug

ously with a slight fall in blood pressure of from 10 to 43 mm Hg the heart rate increased an average of 110 beats per minute the rise persisting for several hours. In every instance the injection of epinephrine (1:50,000) was followed by a fall in blood pressure of from 40 to 50 mm Hg. One of the animals vomited after 400 mgm of the drug had been ingested. No toxic symptoms were noticeable in the remainder of the animals.

*Results Obtained with the Injection and Infusion of 883F into Animals with Experimental Neurogenic Hypertension.* In six cases, 5 mgm per kilogram

TABLE 8  
The effect of the infusion of 888F into normal dogs

NO. OF DOG	DATE OF EXPERIMENT	AMOUNT OF DRUG INFUSED	RATE OF INFUSION	MEAN BLOOD PRESSURE	PRESSURE OF INFUSION OF DRUG	MINUTE VOLUME	HEART RATE	SYSTOLIC DIASTOLIC	SYSTOLIC DIASTOLIC	ARTERIAL-VENTILATION	O <sub>2</sub> INTERNAL-VENTILATION DIFFERENCE	CO-EXTENT OF O <sub>2</sub> UTILIZATION	O <sub>2</sub> CON- SUMPTION	PULMONAL RESISTANCE	dyne cm <sup>-4</sup> sec
11	4/21/44	272 mgm in 150 cc saline	2.5	Before During After	120 118 (30-50)* 100 (53)*	2,650 2,000	60 210 240	197/109 220/84 197/85	44.3 8.3	22.9 24.7	4.3 4.1	18.8 16.6	114 82	3,620 3,760	
17	4/20/44	693 mgm in 150 cc saline	2.5	Before During After	160 140 (30)* 130 (50)*	5,550 180 4,480	90 180 210	250/125 247/122 197/110	61.5 21.4	23.8 24.5	4.2 4.4	17.7 18.0	231 107	2,160 2,320	
14	5/4/44	436 mgm in 150 cc saline	4.5	Before During After	121 96 (Imm.) 110 (50)*	90 220 250	90 184/84 234/107	199/97 184/84 234/107	22.9 27.9	3.2 3.2	14.0 14.0	107 107	3,580		
13	6/27/44	436 mgm in 150 cc saline	4.5	Before During After	160 140 (Imm.) 125 (29)*	3,350 180 2,990	120 180 210	280/138 208/105 148/110	27.9 20.1	3.65 3.65	18.2 18.2	106 106	2,560		

\* Number of minutes after start of the infusion

weight of 883F were injected into dogs with neurogenic hypertension. Table 10 demonstrates a fall in blood pressure of from 60 to 120 mm. Hg, the lowest values being reached in from 3 to 7 minutes. The fall in blood pressure was accompanied by an increase in the heart rate averaging 70 beats per minute. It was evident, therefore, that the injection of 883F into hypertensive animals resulted in a significant fall in blood pressure and an increase in the heart rate.

The results of the infusion of 883F into five hypertensive animals were similar to those obtained with 933F. Shortly after the onset of the infusion the blood pressure fell and the heart rate increased an average of 90 beats per minute (table

TABLE 9  
The effect of oral administration of 883F on normal dogs

DOG	DATE	AMOUNT INJECTED 883F	TIME	HEART RATE	BLOOD PRESSURE
					mm. Hg
14	7/30/44	Control 400 mgm.	10 50	125	140-160
			11 00		
			11 30	100	130
			11 35		
		200 mgm	12 10	200	145
			1 40	240	125
			5 00	100	
18	7/30/44	Control 400 mgm.	11 00	120	110
			11 05		
			11 40	160	110
			11 45		
		200 mgm	12 20	185	100
			1 45	160	115
			5 00	100	
18	7/30/44	Control 400 mgm	11 20	72	140
			11 25		
			12 00	120	158
			12 05		
		200 mgm	12 35	190	115
			1 35	160	115
			5 00	80	

II) This first hypotensive phase lasted for periods ranging from 10 to 25 minutes and was succeeded by a rise in blood pressure of from 15 to 30 minutes duration, the heart rate remaining elevated. From then on the blood pressure declined again, remaining at normal or hypotensive levels for over an hour. In one animal (§ 5) which had received a total of 670 mgm. of 883F the blood pressure continued to fall and the dog died 90 minutes after the infusion had been discontinued.

The hemodynamic alterations during this delayed hypotensive period were similar to those produced by the infusion of 933F (table 11). The heart rate,

the minute volume, and the systolic discharge decreased. The difference between the oxygen content of arterial and mixed venous blood rose significantly in two experiments, remaining unchanged in the remainder. The total peripheral resistance fell in one case, increased slightly in another, and remained constant in a third instance. It was apparent, therefore, that the infusion of 883F, as well as that of 933F, resulted in a biphasic fall in blood pressure. Apparently the hypotensive phase occurring at the end of the infusion was the

TABLE 10  
*Effect of injection of 883F into hypertensive dogs*

DOG	DATE OF EXPERIMENT	AMOUNT OF DRUG INJECTED ( <i>mgm./kilo</i> )		DURING CONTROL PERIOD	AFTER INJECTION	
					mm Hg At lowest blood pressure	mm Hg At highest blood pressure
2	2/15/44	105 mgm in 10 cc saline		Blood Pressure	190	119 (7)*
				Heart Rate	162	202
				Systolic/Diastolic	288/162	188/84
7	2/22/44	100 mgm in 10 cc saline		Blood Pressure	160	52 (5)*
				Heart Rate	240	300
				Systolic/Diastolic	251/122	80/37
5	3/7/44	65 mgm in 10 cc saline		Blood Pressure	171	110 (4)*
				Heart Rate	180	240
				Systolic/Diastolic	242/152	247/105
5	3/14/44	65 mgm in 10 cc saline		Blood Pressure	188	108 (3)*
				Heart Rate	240	300
				Systolic/Diastolic	312/155	167/72
7	3/17/44	100 mgm in 10 cc saline		Blood Pressure	200	120 (3)*
				Heart Rate	250	290
				Systolic/Diastolic		
3	3/24/44	84 mgm in 10 cc saline		Blood Pressure	194	65 (3.6)*
				Heart Rate	180	240
				Systolic/Diastolic	270/140	94/50

\* Number of minutes after administration of drug

result of a decrease in cardiac output in conjunction with decreased or constant total peripheral resistance.

*The Effect of the Oral Administration of 883F on Hypertensive Animals.* In four experiments, 883F in doses ranging from 0.8 to 1.4 gm was given orally to animals with neurogenic hypertension. The total dosage was distributed over a period of from 1½ to 4½ hours. Table 12 shows that the blood pressure declined an average of 75 mm Hg following the ingestion of from 4 to 800 mgm of the drug. In every instance, the heart rate decreased while the blood pressure fell. Five hours after the last dose had been given, epinephrine (1:50,000) caused a

TABLE II  
The effect of the infusion of 85% dioxane derivative drops

NO.	DATE OF EXPERIMENT	AMOUNT OF DROG INFUSED	RATE OF INFUSION cc./min.	MEAN BLOOD PRESSURE mm. Hg	MINUTE VOL. PER MIN.	STERIC DISTANCE mm.	O <sub>2</sub> AMBIENT DIFFUSION DISTANCE mm.	O <sub>2</sub> CON- SUMPTION OF O <sub>2</sub> IN Tissues ml./min.	O <sub>2</sub> CON- SUMPTION OF O <sub>2</sub> IN Tissues ml./min.	STERIC DISTANCE mm.	
										BEFORE	DURING
12	4/ 6/44	341 mgm. in 100 cc. saline	2.5	Before During During During After After	181 80 (1.3) 215 (5.3-18) 183 (20)* 79 (34)* 90 (47)*	3720 270 240 160 125 150	226/142 168/118 250/197 160/127 118/86 128/80	20.7 25.5 20.3 26.4 5.3 20.1	198 20.8 20.1 135 135 19.0	3885	
5	4/ 7/44	670 mgm. in 100 cc. saline	2.6	Before During During During (Died on ta- ble)	170 75 (6.3)* 263 (7.3-22) 170 (22-38) 103 (42)* 60 (71)	4810 180 240 160 3040 120	240/153 128/50 250/191 138/56 25.4	22.7 4.4 20.0 5.1 25.5	220 220 220 155 155	2820	
12	5/ 2/44	361 mgm. in 100 cc. saline	4.5	Before During During After	222 125 (10)* 227 (41)* 95 (63)	5870 250 210 210	308/204 182/72 240/182 158/91	28.7 28.0 12.5 12.5	165 9.3 20.5 20.5	2580	
3	5/12/44	308 mgm. in 110 cc saline	4.5	Before During After	162 117 (11)* 120 (31)*	150 210 150	232/140 178/87 170/110		188 188 188	2860	

Number of minutes after start of the infusion

fall in the blood pressure of from 50 to 60 mm. Hg. In one animal, severe toxic symptoms consisting in vomiting and increased respiratory rate, appeared after the ingestion of 800 mgm of 883F. During the height of the toxic reaction this

TABLE 12  
*The effect of oral administration of 883F on hypertensive dogs*

DOG	DATE	AMOUNT INGESTED 883F	TIME	HEART RATE	BLOOD PRESSURE	
					mm Hg	
3	7/31/44	Control	11 15	180	155-200	
		200 mgm	11 20			
		200 mgm	11 45			
		400 mgm	12 30	180	190	
			2 00	128	100-110	
			3 35	160	130-140	
			4 45	160	140-160	
			5 45	163	150-180	
7	7/26/44	Control	10 15	200	200-140	
		200 mgm	10 30			
		200 mgm	10 55			
		400 mgm	11 45	200	160	
			12 45	180	100-105	
			3 00	120	150	
			4 00		180	
2	7/31/44	Control	11 05	180	180-155	
		200 mgm	11 07			
		200 mgm	11 35			
		400 mgm	12 40	172	145	
			2 30	200	185	
			3 20	110	120	
			4 15	150	105	
			5 30	162	135	
			6 30		175	
7	7/31/44	Control	10 55	160-180		180-140
		200 mgm	11 00			
		200 mgm	11 30			
		200 mgm	12 10	172	135	
			12 15			
			2 30	160	110-125	
			4 30	200	115	
			5 15	190	120-140	
			5 35	168	130	
			6 30		160	

animal appeared listless and apathetic. In the remaining three dogs toxic symptoms were not observed. Although alterations in the cardiac output and the total peripheral resistance were not followed in these experiments, the assumption might be ventured that the fall in blood pressure was the result of a

decrease in the cardiac output. This conclusion was supported by the observation that the heart rate of the hypertensive animal decreased during the administration of the drug.

*The Infusion of Saline into Animals with Neurogenic Hypertension.* Since the possibility existed that the hypotensive effects observed during the administration of the dioxanes represented normal fluctuations in the blood pressure, which were commonly encountered in animals with neurogenic hypertension (8) three experiments were performed in which the effect of the infusion of 150 cc of normal saline was investigated. As seen in table 13, a slight fall in blood pressure of from 15 to 30 mm Hg occurred during the infusion of the saline solution. This fall, however, was less marked than that observed during and following the infusion of the dioxane derivatives. The blood pressure during the infusion of saline fell for a period of 4 to 5 minutes only in contrast to the

TABLE 13  
Effect of the infusion of saline into hypertensive dogs (rate 4.5 cc./min.)

DOG	DATE OF EXPERIMENT	AMOUNT OF SALINE INFUSED		DURING CONTROL PERIOD	AFTER INFUSION	
					At lowest blood pressure	At highest blood pressure
2	5/10/44	150	Blood Pressure	180 mm. Hg	190 (15)	190 (20)
			Heart Rate	180	180	185
			Systolic/Diastolic	258/154	271/159	275/160
12	5/20/44	150	Blood Pressure	180 mm. Hg	160 (20)	214 (34)
			Heart Rate	240	220	210
			Systolic/Diastolic	250/162	204/140	308/182
7	6/24/44	150	Blood Pressure	160 mm. Hg	145 (20)	214 (25)
			Heart Rate	180	180	180
			Systolic/Diastolic	215/126	180/106	200/115

Number of minutes after the administration of the drug

hypotension produced by the dioxane derivatives, which persisted for at least 40 minutes. It was apparent, therefore, that the decline of the blood pressure observed during and following the injection and ingestion of 883 and 933F was not caused by normal fluctuation of the blood pressure but was the result of the dioxanes on the circulatory system.

*The Action of 883F on Animals with Renal Hypertension.* Thanks to the co-operation of Dr. Harry Goldblatt the effect of the injection and the infusion of 883F into dogs with renal hypertension could be incorporated in this report. The technique employed by Dr. Goldblatt was identical with that used in the experiments reported above. 883F was injected in dosage of 5 mgm. per kilogram weight into two animals with renal hypertension. No change in the blood pressure occurred during or following the injection. Toxic symptoms were entirely absent. In another series 5 mgm. of 883F per kilogram weight were

dissolved in 150 cc of saline and infused into two dogs with renal hypertension at a rate of 2.5 cc per minute. In both cases, a slight but significant elevation of the blood pressure, ranging from 20 to 25 mm Hg, and an increase in the heart rate averaging 100 beats per minute, were observed. These results demonstrated that the injection or infusion of 883F into animals with renal hypertension failed to elicit a fall in the blood pressure. They contrast with those obtained on animals with chronic neurogenic hypertension, in which the administration of 883F produced a marked reduction of the blood pressure.

**DISCUSSION** The experiments reported in this paper show that the injection of 933F into normal animals produces a slight rise in the blood pressure and an increase in the heart rate, changes which have previously been described by Handovsky (5). This tachycardia has also been described by Katz (10). The infusion of this compound has no effect on the blood pressure, but produces a marked tachycardia. The oral administration of 933 and 883F, as well as the injection and the infusion of 883F, elicit a slight fall in the blood pressure and a significant increase in the heart rate of normotensive animals. The hemodynamic changes produced by the infusion of these dioxanes into normal animals consist in a moderate fall of the minute volume, and a decline in the systolic discharge. After the infusion of 883 and 933F, the total peripheral resistance does not change except in one instance, in which it falls following the administration of 883F.

The action of the Fourneau drugs on normotensive animals differs from that observed in animals with chronic neurogenic hypertension. In dogs with neurogenic hypertension, the injection of 883 and 933F is followed by a marked fall in the blood pressure, accompanied by an increase in the heart rate. Similar observations have been made by Heymans and Bouckaert (7). Hypotension and tachycardia are also present during the early stages of the intravenous infusion. In these cases, however, the fall in blood pressure is followed by a rise, and finally toward the end of the infusion by a decline in blood pressure and the heart rate. The hemodynamic alterations during this delayed hypotensive phase consist in a fall in the minute volume and in the systolic discharge. The total peripheral resistance declines after the infusion of 933F. Following the infusion of 883F, it decreases in two cases, rises slightly in another, and remains constant in a fourth instance. When the drugs are administered orally to animals with sectioned moderator nerves, tachycardia and a rise of the blood pressure above its control values are not observed. After ingestion of from 0.4 to 0.8 gm, both the heart rate and the blood pressure decline.

These studies reveal that in the normotensive animal the cardiac output is maintained by an increase in the heart rate. In animals with chronic neurogenic hypertension, however, the heart rate decreases toward the end of the infusion, consequently the minute volume of these animals falls. It is impossible to ascertain from the experiments reported in this paper whether these cardiac changes are caused by the action of the dioxanes on the vasomotor center or on the heart muscle. The work of Handovsky indicates that the rise

in blood pressure and the increase in the heart rate is caused by central stimulation (5). The findings of Vleeschhouwer and Heymans suggest that the depressor action observed in dogs with chronic neurogenic hypertension also results from direct action of the dioxanes on the vasomotor center (6).

The results obtained in control experiments in which normal saline is infused instead of the dioxane preparations indicate that the fall in blood pressure which follows the administration of the dioxanes is the result of these substances and does not represent normal fluctuations in pressure, which are commonly encountered in neurogenic hypertension. This conclusion is further substantiated by the following observations (1) that the hypotension which follows the administration of 883 and 933F persists for periods ranging from 3 minutes to several hours, and (2) that excitatory stimuli, such as repeated puncture of the femoral artery fail to reestablish the hypertension. In contradistinction normal fluctuations of the blood pressure in animals with severed moderator nerves are of short duration and the slightest excitatory stimulus is usually sufficient to raise the blood pressure (8). The reversal of the adrenaline effect observed following oral administration of the Fourneau compounds indicates that the drugs are also active when they are administered by mouth.

It is uncertain at the present moment whether 883 and 933F possess any value in the treatment of essential hypertension. The experiments reported in this paper as well as the work of Kats (10) and that of Bovet and Simon (4) show that 883 and 933F are highly toxic when they are administered in large doses. When given in smaller still effective quantities, however, their toxicity is slight or absent. The present observations show no toxic symptoms after single intravenous injections of from 2.5 to 5 mgm of either compound. Slight salivation and a moderate increase in the respiratory rate are noticeable during and following the infusion of from 50 to 90 mgm of both compounds. During oral administration occasional vomiting occurs and some of the animals appear to be listless and apathetic. Other dioxanes including 1071 and 1072F whose central action is small (4), have little effect on the blood pressure of dogs with chronic neurogenic hypertension (11).

The increase in the heart rate which occurs during single injections and at the onset of intravenous infusions, however, constitutes a serious objection to the parenteral administration of these compounds. Since tachycardia is absent after oral ingestion, this route would seem to be the method of choice for the administration of 883 and 933F in man.

The experiments of Dr Goldblatt, which reveal that 883F fails to lower the blood pressure of animals with renal hypertension suggest the possible use of dioxane derivatives and other related compounds in the differentiation of hypertension of neurogenic from that of renal origin in man. Such a differentiation is at present only possible by arresting the sympathetic outflow through spinal block (12). The use of the Fourneau substances or similar compounds might possibly furnish additional information on this subject, which is of great clinical and theoretical importance.

## SUMMARY

1 The effect of the intravenous injection, infusion, and oral administration of two dioxane derivatives, 883 and 933F, on normal unanesthetized dogs, and on animals with chronic neurogenic hypertension, was studied. In addition, the action of 883F on animals with renal hypertension was observed.

2 In the normal animal, the injection of 933F produced transient hypertension, the infusion of this compound was without effect on the blood pressure, while its oral administration resulted in a slight fall. The heart rate increased after both intravenous and oral administration.

3 The intravenous injection and infusion of 883F into normotensive animals as well as the oral ingestion of this compound, was succeeded by a slight fall in blood pressure and by a significant increase in the heart rate.

4 The hemodynamic changes observed in normal animals during the infusion of 933 and 883F, consisted in a decline of the systolic discharge and an increase in the heart rate. The minute volume was maintained or fell only slightly. The total peripheral resistance remained unchanged.

5 In animals with chronic neurogenic hypertension, the injection of 883 and 933F elicited a transient fall in blood pressure and an increase in the heart rate. The infusion of these compounds was followed by a decline in blood pressure, succeeded by a short period of hypertension with tachycardia. Toward the end of the infusion the heart rate and blood pressure declined. The oral administration of the two dioxanes resulted in a fall in blood pressure and a decrease in the heart rate, the effect persisting for several hours.

6 The hemodynamic alterations observed during the infusion of 933 and 883F into animals with sectioned moderator nerves consisted in a fall in systolic discharge, accompanied by a decrease in the pulse rate and in the minute volume.

7 The infusion of saline into animals with chronic neurogenic hypertension did not result in a prolonged decrease in the blood pressure or the heart rate.

8 The injection and infusion of 883F failed to lower the blood pressure of animals with chronic renal hypertension.

9 The possible application of these findings in the treatment of essential hypertension and in the differentiation of essential from renal hypertension is discussed.

We are indebted to Miss Christine Waples, Dr T Finney, and Mr D Whitener for their cooperation and assistance. We are especially grateful to Dr W T Longcope for his advice and inspiration and to Dr G Harrop for supplying us with the dioxane derivatives.

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## ANESTHESIA XVIII

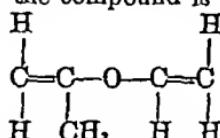
### A COMPARATIVE STUDY OF PROPETHYLENE AND CYCLOPROPANE ON CARDIAC AUTOMATICITY<sup>1</sup>

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Propethylene is a volatile anesthetic agent which was shown to possess promising anesthetic properties in the mouse, rat, dog and *Macacus rhesus* monkey by Krantz et al (1) Chemically the compound is isopropenyl vinyl oxide, which



has the following composition, Davis and Krantz (2) con-

firmed its anesthetic usefulness in man. Evans et al (3) showed that prolonged and repeated anesthesias with propethylene were neither hepatotoxic nor nephrotoxic in the mouse, rat and dog. Evans et al (4) developed a method to estimate propethylene quantitatively in the circulating blood and found anesthetic concentrations in the dog and monkey to be of the order of magnitude of 30 to 40 mg per cent. Blood gases and cardiac rhythm were found to be essentially unaltered by anesthesia with propethylene in the dog even at respiratory arrest by Evans and Krantz (5).

Meek, Hathaway and Orth (6) showed that arrhythmias frequently occurred in the dog's heart under cyclopropane anesthesia when the automatic tissue of the heart was sensitized by intravenous injections of epinephrine. In these studies, we have compared the form and rhythm of the electrocardiographic tracings of the dog and *Macacus rhesus* monkey under cyclopropane and propethylene anesthesias respectively, before and during the injection of epinephrine.

**METHOD OF STUDY** An electrocardiographic tracing Lead II was made of a normal dog or monkey. Epinephrine hydrochloride solution, 1:100,000 (preserved with sodium bisulfite) was injected intravenously, 0.01 mg./kg. diluted in each case to a 5 cc. volume. The injection was made during 50 seconds. At the end of 40 seconds at the height of the effect as determined by a cardioscope, another electrocardiogram Lead II was taken during 20 seconds. Each animal was subsequently anesthetized with propethylene and cyclopropane respectively, using the closed circuit method with oxygen. In the third plane of surgical anesthesia another electrocardiogram was taken and then epinephrine was injected as set forth in the foregoing description and another record taken. The results are set forth in tables 1 and 2.

Typical electrocardiograms from each table are shown in charts 1 and 2.

<sup>1</sup> The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Co. of Cleveland, Ohio.

<sup>2</sup> Associate Professor of Physiology.

**DISCUSSION** The work of Orth Stutzman and Meek (7) showed that cyclopropane greatly enhanced the activity of epinephrine on the automatic tissues of the dog's heart. In their work in control animals epinephrine produced only escape phenomena but under the anesthetic multifocal ventricular tachycardia occurred in practically all experiments. On this as a basis Orth stated that

TABLE 1  
*Dog and monkey propylene anesthetics with epinephrine*

	NO. ANIMALS	INITIAL RATE INCREASED	INITIAL RATE DECREASED	A-V BLOCK	VENTRICULAR EXTRASYNCHRONY	VENTRICULAR EXTRASYNCHRONY	A-V NODAL ARRHYTHMIA	SLOW VENTRICULAR RATE	VENTRICULAR TACHYCARDIA	VENTRICULAR STIMULATION	SPONT. ARRHYTHMIA	INVERTED T-WAVE
Normal dog	10										2	
Dog with epinephrine	10		7	2			2	2			6	1
Propylene anesthesia	10	8										3
Propylene anesthesia with epinephrine	10	2	2				1					
Normal monkey	3											
Monkey with epinephrine	3		3	1								2
Propylene anesthesia	3	2										
Propylene anesthesia with epinephrine	3	2										

TABLE 2  
*Dog and monkey cyclopropane anesthetics with epinephrine*

	NO. ANIMALS	INITIAL RATE INCREASED	INITIAL RATE DECREASED	A-V BLOCK	VENTRICULAR EXTRASYNCHRONY	VENTRICULAR EXTRASYNCHRONY	A-V NODAL ARRHYTHMIA	SLOW VENTRICULAR RATE	VENTRICULAR TACHYCARDIA	VENTRICULAR STIMULATION	SPONT. ARRHYTHMIA	INVERTED T-WAVE
Normal dog	10											
Dog with epinephrine	10		9	5			4				8	1
Cyclopropane anesthesia	10	5	3									
Cyclopropane anesthesia with epinephrine	10	4	3	3		1	5	1	2	1	5	3
Normal monkey	3											
Monkey with epinephrine	3		3									1
Cyclopropane anesthesia	3		2									
Cyclopropane anesthesia with epinephrine	3		1	1	1	1	1	1			2	1

epinephrine was interdicted in cyclopropane anesthesia. Using similar sympathomimetic amines, they observed that this effect was specific for compounds of the series which like epinephrine contained the catechol nucleus those containing the phenyl or phenol nucleus did not elicit the response. Allen et al (8) showed that sympatholytic agents like ergotamine and yohimbine prevented the

cyclopropane-epinephrine arrhythmias Wright (9) suggested that this action was centric and depended upon the depression of certain motor nuclei by ergot amine Thienes and Greeley (10) in a review of this entire subject, hold the view that an "intact hypothalamus, rather than the intrinsic cardiac tissues, is the site of the action of cyclopropane in this regard."

In this series of experiments, we have confirmed the findings of previous investigators, namely, that injections of epinephrine in the dog under cyclopropane

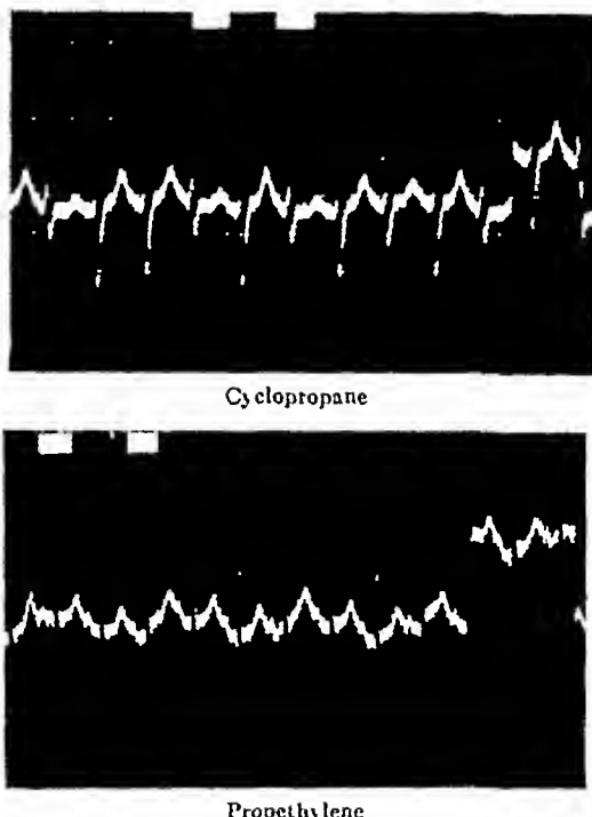


CHART 1 MONKEY'S HEART UNDER PROPYYLENE AND CYCLOPROPANE RESPECTIVELY UPON THE INJECTION OF EPINEPHRINE LEAD II

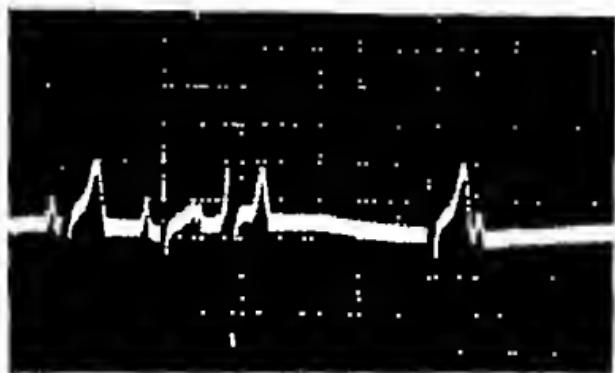
anesthesia produced in a large percentage of animals arrhythmias of a serious character Orth et al (11) obtained ventricular fibrillation in 5 out of 20 dogs, we found this to occur in one of our series of 10

We have extended the work on cyclopropane to the *Macacus rhesus* monkey and found that in this species, as in the dog, epinephrine in animals under cyclopropane anesthesia produced serious cardiac disturbances, including a bundle branch block in one of the 3 animals

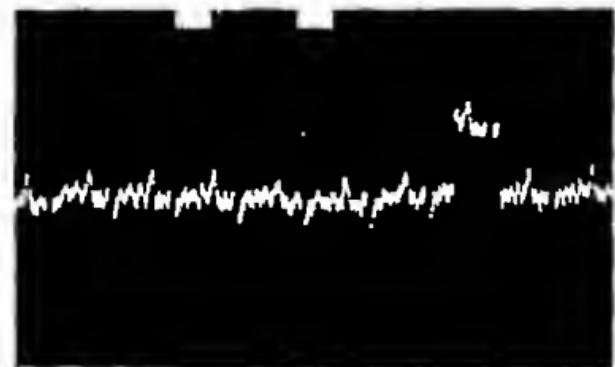
Table 1 shows that the heart is not sensitized to epinephrine under propylene anesthesia. In this series, this holds for the dog and the monkey. Propylene appears to have a greater capacity than cyclopropane to increase

the heart rate. In 20 to 30 per cent of the anesthesias in the dog in this series, and in a former series (5) the T wave in Lead II is inverted. It is of special interest that in our studies with cyprethylene ether (12) an isomer of propethylene

having the structure  $\text{H}_2\text{C} \begin{array}{c} \text{CH}_2 \\ \diagdown \\ \text{C} \end{array} \text{O} \begin{array}{c} \text{H} \\ \diagup \\ \text{C} \end{array} \begin{array}{c} \text{H} \\ \diagdown \\ \text{H} \end{array}$  in a series of 15 monkey an-



Cyclopropane



Propethylene

CHART 2 Dog's Heart Under Propethylene and Cyclopropane Respectively Upon the Injection of Epinephrine Lead II

anesthesia we observed 2 branch bundle blocks and in others arrhythmias. This seems to indicate that the 3 membered cyclopropane ring in the molecule is responsible for eliciting cardiac arrhythmias.

#### CONCLUSIONS

1. The sensitization of the heart to epinephrine under cyclopropane anesthesia in the dog has been confirmed and extended to the Macacus rhesus monkey.
2. Cardiac tissue is not rendered sensitive to epinephrine under propethylene anesthesia in the dog and monkey.

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# STUDIES ON THE ANTAGONISM OF SODIUM SUCCINATE TO BARBITURATE DEPRESSION

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In a recent publication Soakin and Taubehaus (1) proposed the use of sodium succinate as an antidote to barbiturate poisoning. Its effectiveness was ascribed to the fact that the oxidation of succinate is not inhibited by the presence of barbiturates as is that of glucose lactate and pyruvate (2). Sodium succinate therefore was assumed to furnish an oxidizable substrate until the barbiturate could be destroyed or excreted by the body. They found that the preanesthetic intramuscular administration of 100 mg. sodium succinate per 100 grams body weight protected rats against the fatal effects of sodium pentobarbital (8.5 mg per 100 grams body weight, given intraperitoneally) in 85 per cent of the cases, whereas in the control group only 45 per cent of the animals recovered.

These authors report succinate to be effective also in controlling the duration of anesthesia in rats given 2.5 mg sodium pentobarbital per 100 grams body weight. The median duration of anesthesia was reduced to one-half of the control value by the administration of 5 mg. sodium succinate per 100 grams while 37.5 mg. per 100 grams reduced the duration to one-third of the control value.

Landy, Hansen and Phillips (3) were unable to confirm the above results in regard to control of the duration of barbiturate anesthesia.

According to Beyer and Latven (4, 5) injection of 150 mg. sodium succinate per kg. intramuscularly into mice immediately following loss of the righting reflex failed to shorten the duration of anesthesia produced by sodium pentobarbital (80 mg. per kg., intraperitoneally) but when 1000 mg. sodium succinate per kg. was administered the significant difference between the treated and control mice was 5.25 (2.0 or above indicates a significant difference). The effect of succinate on duration of anesthesia in rats was found to be less than that reported by Soakin and Taubehaus.

The present experiments were undertaken for the purpose of testing further the properties of sodium succinate as an analeptic.

**EXPERIMENTAL. Protection against pentobarbital poisoning.** That portion of the work of Soakin and Taubehaus (1) dealing with protection against sodium pentobarbital poisoning was first repeated. Male hooded rats were used. Their average weight was 328 grams considerably greater than the weight of the rats used by Soakin and Taubehaus. They were given no food but were allowed free access to water during the 16 hours preceding the experiments. Forty rats were injected intramuscularly with 100 mg. sodium succinate per 100 grams body weight, each dose being diluted with distilled water to make 1 cc. in volume. The dilutions were made from a freshly prepared 50 per cent stock solution. This

treatment was followed after 15 minutes by the intraperitoneal administration of 8.5 mg sodium pentobarbital (Nembutal<sup>1</sup>) per 100 grams body weight as an 8.5 per cent aqueous solution. In an equal number of control rats only the pentobarbital was injected. A comparison of results in the experimental and control groups is given in table 1. Of the 40 control rats 21 died (52.5 per cent) while among those treated with sodium succinate there were 8 deaths (20 per cent). This difference (32.5 per cent) is 3.2 times the standard error and therefore significant. The time elapsing before death or recovery of the individual animals is also indicated in the table. Median values for recovery time of control and experimental animals were 347 minutes and 320.5 minutes respectively. Median time of death was 75 minutes in the control group and 146.5 minutes in the experimental group. Duration of anesthesia in our experiments was therefore much longer than that in the experiments of Soskin and Taubenhau<sup>s</sup>, a circumstance perhaps due to weight differences (6).

In the course of these experiments it was observed that stock solutions of sodium succinate left standing for a number of days lost much of their analeptic effect. Thus fatalities from sodium pentobarbital among rats treated with old succinate solutions occurred in approximately the same proportions as among control rats. The following experiment was designed to investigate this aging factor.

Since hooded rats were no longer available, male albino rats averaging 314 grams in weight were used. Four groups of forty animals were established. All animals received 8.5 mg sodium pentobarbital per 100 grams body weight intraperitoneally. This was preceded, by 15 minutes, by intramuscular injection of 1 cc isotonic solution of sodium chloride in Group A (control animals) and by intramuscular administration in the three remaining groups of 100 mg. sodium succinate per 100 grams body weight given in 1 cc doses diluted from the following 50 per cent solutions. Group B, freshly prepared sodium succinate solution, Group C, sodium succinate solution prepared with sterile precautions 10 days previously, and Group D, sodium succinate solution prepared without sterile precautions 10 days previously and exposed to the air for one hour each day until use. Upon bacteriological examination the latter solution was found to be uncontaminated by either aerobic or anaerobic forms in spite of lack of sterile handling.

It is apparent from table 2 that there is a reduction in the protective power of succinate solutions upon standing. Fifty per cent of the control animals died and 30 per cent of the animals treated with fresh succinate died. In each of the groups injected with aged succinate solution 45 per cent of the animals died. The probability that a true difference exists in regard to fatalities between the controls and the animals injected with fresh succinate is approximately ninety-three in one hundred. The probability that a true difference exists between the fatalities in the controls and in the animals treated with old sterile and old "non-sterile" solutions is sixty-four in one hundred and fifty-five in one hundred respectively.

<sup>1</sup> Supplied through the kindness of Dr R K Richards of Abbott Laboratories

The first two sets of animals in this experiment constitute a repetition, except for the variety of the experimental animal, of the work with hooded rats re-

TABLE 1

*Duration of anesthesia in rats given 8.5 mg sodium pentobarbital per 100 gram body weight intraperitoneally*

Treated rats were given 100 mg sodium succinate per 100 grams body weight intramuscularly 15 minutes prior to administration of the anesthetic.

CONTROL		TREATED	
Recovered	Died	Recovered	Died
min.	min.	min.	min.
160	41	249	49
164	42	250	76
266	44	266	92
291	55	266	117
298	59	269	178
300	60	283	178
332	62	291	251
340	62	294	269
341	67	295	
347	76	304	
359	78	305	
362	80	309	
363	84	310	
377	89	313	
396	91	317	
407	101	317	
428	101	324	
525	127	325	
740	140	335	
	170	339	
	165	348	
		352	
		366	
		374	
		383	
		384	
		390	
		401	
		422	
		430	
		470	
		470	
Median	347	78	320.5
Mean	357.7	86.4	335.6
			148.5
			151.0

ported above, it may be seen that succinate afforded less protection to the albino rats in this experiment than to the hooded rats of the first experiment.

The effect of succinate on anesthesia in albino mice was investigated in two

experiments similar to the first. Male animals averaging 23.2 grams in weight were used. Sodium succinate was administered by two routes, subcutaneously in the first series and intravenously in the second.

To each of 100 mice was given 0.15 mg sodium pentobarbital per gram body weight, in 1.5 per cent solution, intraperitoneally. One-half of these animals were given 1 mg sodium succinate per gram body weight subcutaneously as a 10 per cent solution 15 minutes prior to the administration of the anesthetic. Of these 50 mice 41 died (82 per cent) and of the 50 control mice 37 died (74 per cent). The difference between the groups is of no statistical significance. Median death times (table 3) were 14 minutes for the control group and 16 minutes for the experimental group. Mean death times were 16.3 minutes and 20.3 minutes for the two groups respectively. Median recovery times were 287 minutes in the control group and 169 minutes in the experimental group.

TABLE 2

*Effect of aging on analeptic property of sodium succinate solutions in albino rats*

Succinate (100 mg per 100 grams body weight) injected intramuscularly 15 minutes prior to intraperitoneal administration of 8.5 mg sodium pentobarbital per 100 grams body weight.

	NUMBER OF ANI- MALS	NUMBER OF SUR- VIVALS	PER CENT OF SUR- VIVALS	NUMBER OF DEATHS	PER CENT OF DEATHS	MEAN RECOV- ERY TIME	MEDIAN RECOV- ERY TIME	MEAN DEATH TIME	MEDIAN DEATH TIME
Control	40	20	50	20	50	335	334	148.6	137
Freshly prepared sodium succinate	40	23	70	12	30	353.8	316	155	171
10-day old sterile sodium succinate	40	22	55	18	45	323.5	314.5	204.4	173
10-day old "non sterile" sodium succinate	40	22	55	18	45	302.5	293	149.2	144

Mean recovery times were 255.8 minutes for the controls and 191.2 minutes for the treated mice.

Sodium succinate was administered intravenously to 50 of a second series of 100 mice. In this instance 1 mg per gram body weight was injected into the tail vein approximately three minutes after intraperitoneal administration of 0.15 mg sodium pentobarbital per gram body weight. There were 42 deaths (84 per cent) among the control animals and 36 deaths (72 per cent) in the treated group. This difference is not significant statistically. Median death times were 13 minutes for the control group and 16 minutes for the treated group. Mean death times in the two groups were 15.1 minutes and 16.5 minutes. Median recovery times were 222 minutes for the control animals and 203 minutes for the treated animals. Mean recovery times were 230.1 minutes and 188.4 minutes respectively for these two groups.

We were thus unable to demonstrate a reduction in the number of fatalities in mice by the administration of sodium succinate subcutaneously or intravenously.

In the preceding experiments there was displayed a tendency among surviving animals for the duration of anesthesia to be shortened by the presence of succinato and for deaths to be delayed in the treated cases. However the fact that recovery time could be measured for only part of the group those surviving the experiment, makes it impossible to treat the data statistically.

*Duration of anesthesia.* For measuring the effect of succinate upon duration of pentobarbital anesthesia, male rabbits (1.25 to 2.15 kg.) were used. Twelve control and 12 experimental animals were used in one series in which a near

TABLE 3

*Effect of sodium succinate on mortality from 0.15 mg. sodium pentobarbital per gram body weight in mice*

Sodium succinate 1 mg. per gram was given subcutaneously 15 minutes prior to administration of the anesthetic in the first treated group and intravenously 3 minutes after administration of the anesthetic in the second treated group.

	NUMBER OF ANIMALS	NUMBER OF SUB-SURVIVALS	PER CENT SUB-SURVIVALS	NUMBER OF DEATHS	PER CENT DEATHS	MEAN RECOVERY TIME	MEDIAN RECOVERY TIME	MEAN DEATH TIME	MEDIAN DEATH TIME
1 Control	50	13	26	37	74	255.8	287	16.3	14
Succinate subcutaneously	50	9	18	41	82	191.2	189	20.3	16
2 Control	50	8	16	42	84	230.1	222	15.1	13
Succinate intravenously	50	14	28	36	72	188.4	203	16.5	16

TABLE 4

*Effect of sodium succinate on duration of anesthesia in rabbits anesthetized with sodium pentobarbital 50 mg./kg. body weight intravenously*

	NUMBER OF ANIMALS	RECOVERY OF ERECTED REFLEX			RECOVERY OF ABILITY TO WALK		
		Average time	Standard deviation	Standard error	Average time	Standard deviation	Standard error
Control	12	140.8	26.8	8.1	190.1	41.9	11.7
Treated (sodium succinate 1 gram/kg.)	12	111.4	43.2	13.0	140.8	41.3	12.4

fatal dose (50 mg. per kg. body weight) of the anesthetic was given intravenously. This was followed after 20 minutes, in the experimental group by slow intravenous injection (1 cc. per minute) of 1 gram sodium succinate per kg. each dose diluted to 20 cc. and in the control group by injection of an equal volume of isotonic solution of sodium chloride. (Large doses of succinate administered by rapid intravenous injection result in pulmonary hemorrhage and death.) The duration of anesthesia was determined by establishing two points the return of the righting reflex and the somewhat later return of voluntary walking movements. As shown in table 4 mean recovery time as measured by the return of the righting

reflex was 140.8 minutes for the control group and 111.4 minutes for the treated animals. Mean times for recovery of ability to walk were 190.1 minutes and 140.8 minutes for the two groups respectively. The difference between treated and untreated animals in regard to recovery of righting reflex was shown to be on the borderline of statistical significance (a *t* value of 2.00 was obtained where 2.07 represents a 1.20 probability), the difference in respect to return of voluntary walking was definitely significant (*t* is 2.96 where 2.82 represents a 1.100 probability).

In order to compare the analeptic properties of succinate with those of the most commonly accepted antidote for barbiturate poisoning, picrotoxin (7), another set of experiments was conducted. It was further supposed that in view of the different modes of action of picrotoxin and succinate, their simultaneous administration might result in summation of effect. Four groups of 12 male rabbits each (1.28 to 1.81 kg) were used, all were given 35 mg sodium

TABLE 5

*Effect of sodium succinate and of picrotoxin on duration of anesthesia in rabbits anesthetized with sodium pentobarbital 35 mg/kg body weight intravenously*

	NUMBER OF ANIMALS	RECOVERY OF RIGHTING REFLEX			RECOVERY OF PLACEMENT REACTIONS			RECOVERY OF ABILITY TO WALK		
		Average time	Stand- ard de- viation	Stand- ard error	Average time	Stand- ard de- viation	Stand- ard error	Average time	Stand- ard de- viation	Stand- ard error
Control	12	106.5	34.7	10.5	134.4	52.3	15.7	137.3	50.2	15.1
Sodium succinate 1 gram/kg	12	81.8	26.6	8.0	115.6	43.8	13.2	122.0	45.0	13.6
Picrotoxin 2 mg/kg	12	56.0	16.4	4.9	86.3	29.2	8.8	87.9	30.5	9.2
Sodium succinate 1 gram/kg plus pic- rotoxin 2 mg/kg	12	53.8	12.5	3.8	76.2	30.7	9.3	74.0	23.5	7.1

pentobarbital per kg body weight intravenously. Twenty minutes after onset of anesthesia animals of the various groups were given (a) sodium succinate, 1 gram per kg intravenously, (b) picrotoxin, 2 mg per kg intravenously, (c) sodium succinate, 1 gram per kg, followed by picrotoxin, 2 mg per kg, intravenously, and (d) controls, isotonic solution of sodium chloride. Total volume administered was adjusted to 20 cc in all cases by the injection of isotonic saline. The succinate was injected at the rate of 1 cc per minute. Criteria for duration of anesthesia were recovery of the righting reflex, recovery of placement reactions and walking. (Two placement reactions were considered. When the dorsum of a normal rabbit's foot is touched to the edge of a table the animal immediately lifts his foot onto the table. When his chin is touched to the table-top, the animal being otherwise suspended by the experimenter, he reacts by placing both forefeet upon the table. In our experiments these two reflexes returned practically simultaneously.)

Average duration of anesthesia for the various groups as judged by these criteria is recorded in table 5. It may be seen that picrotoxin in the dosage used is more effective than sodium succinate in shortening duration of pentobarbital anesthesia, and that there is little summation in effect when the two drugs are used simultaneously. In this series the *t* value for difference in mean time for recovery of the righting reflex between control and succinate treated groups prove to be less than significant as did also that calculated for difference between these two groups in regard to recovery of the placement reactions and the return of voluntary walking. The *t* values for difference in mean recovery time between the control group and the group given picrotoxin alone were 4.55, 2.76 and 2.94 when measured by return of righting reflex, placement reaction and walking respectively. All three figures are significant. Comparison of the controls with animals of the group treated with both drugs simultaneously yields highly significant *t* values, 4.94 when measured by return of righting reflex, 3.32 when measured by return of placement reactions and 3.96 when measured by the recovery of ability to walk. No significant difference existed between the results from the use of picrotoxin alone and those from the combined use of picrotoxin and sodium succinate.

The pattern of recovery from anesthesia was essentially the same in all animals regardless of treatment, differing only in rate of recovery. No convulsions were encountered in the use of picrotoxin.

#### SUMMARY

1 Repetition of the experiment of Soekin and Taubenhause concerning the use of sodium succinate as an antidote to barbiturate poisoning in hooded rats yielded results similar in nature but somewhat less striking than those obtained by these workers. The preadministration of 1 gram sodium succinate per kg body weight protected 80 per cent of the treated rats against death from 8.5 mg sodium pentobarbital per kg, whereas 47.5 per cent of the control animals survived.

2 In an identical experiment with albino rats, 70 per cent of the treated rats recovered while 50 per cent of the control animals recovered.

3 Sodium succinate solutions were found to become less effective upon aging. The number of survivals among albino rats treated with 10 day old solutions of sodium succinate scarcely exceeded those among control rats.

4 No significant difference in death rate was noted from sodium pentobarbital administration between control and treated mice when sodium succinate was administered either subcutaneously or intravenously.

5 Sodium succinate was shown to shorten duration of sodium pentobarbital anesthesia in rabbits to a limited extent. It was far less effective than a moderate dose of picrotoxin.

6 Little additive analeptic effect was obtained by the simultaneous administration of sodium succinate and picrotoxin to rabbits previously given sodium pentobarbital.

The authors are indebted to Dr Ernst Fischer, Department of Physical Medicine, for advice on the statistical treatment of the data, and to Dr J D Reid, Department of Bacteriology, for making anaerobic cultures on our solutions

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# DISTRIBUTION OF TUNGSTEN IN THE RAT FOLLOWING INGESTION OF TUNGSTEN COMPOUNDS

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In an attempt to utilize tungsten or one of its compounds for hæmography and hepatography information was needed concerning the distribution of tungsten in various organs of the rat following oral ingestion of tungsten compounds. Earlier experiments (1) indicated that sodium tungstate or tungstic oxide in concentrations equivalent to 0.1 per cent W and ammonium paratungstate equivalent to 0.5 per cent W when fed to rats *ad libitum* over a period of 70 days were only slightly toxic as evidenced by a small retardation in growth.

Bernstein-Kohan (2) fed sodium tungstate to a single rabbit and tungsten metal to a single cock. After sacrificing the animals, a crude colorimetric method showed no tungsten in the liver or kidney of either animal, but 2.25 per cent of the solid material of the rabbit's bone was found to be tungsten. These few experiments are inconclusive and indicate the need of further study.

**EXPERIMENTAL.** Thirty-seven day old rats were placed on a basal diet of ground Purina dog chow in which the finely-ground tungsten compounds had been incorporated. For each experimental diet, 2 female and 2 male rats were used with the sexes separately caged. Diet and water were given *ad libitum*. At the end of approximately one hundred days, the rats were killed by a sharp blow upon the back of the neck. Before removing the tissues, the skin was scrubbed and washed well.

The tissues of both rats on the same diet were combined, weighed, rinsed free of blood and prepared for analysis. The method of Aull and Kinard (3) was used for the determination of the tungsten.

The experimental diets were as follows:

Diet A. Purina dog chow (control). Diet B. 0.1262 per cent tungstic oxide (0.1 per cent W) in Purina dog chow. Diet C. 0.1795 per cent sodium tungstate (0.1 per cent W) in Purina dog chow. Diet D. 0.8912 per cent ammonium paratungstate (0.5 per cent W) in Purina dog chow. Diet E. 2.0 per cent tungsten metal (2.0 per cent W) in Purina dog chow. Diet F. 10.0 per cent tungsten metal (10.0 per cent W) in Purina dog chow. Diet G. 10.0 per cent purified tungsten metal<sup>1</sup> (10.0 per cent W) in Purina dog chow.

**RESULTS.** The results of the analysis of bone, skin, and spleen are presented in table 1 with quantities of less than 1.0 mgm. per cent of W being reported as a trace. The other organs or tissues analyzed gave variable results which are

<sup>1</sup> Tungsten Metal used in Diet G was purified further by washing with 0.1 N HCl, wash free of acid, washing several times with 0.1 N NaOH, washing free of the last trace of alkali and then drying.

not included in the table but which are placed in three general groups and summarized below.

*Brain, Heart and Uterus* Generally negative, with a single exception in each case. A trace of tungsten was found in the brain of the males on Diet D and in the heart of the females on Diet G. The uterus of the animals on Diet C contained 3 mgm per cent of W.

*Lung, Muscle and Testis* Positive in approximately one half of the animals tested, but in no case was more than a trace of tungsten found. No relation was observed between diet or sex and the presence or absence of tungsten.

*Blood, Kidney and Liver* Generally positive, but not more than a trace of W. No exception to this generality was observed in the case of liver. In the blood of the females on Diets C and D no tungsten was found. No tungsten was found in the kidneys of the females on Diet B, but those of the females on Diet D contained 2 mgm per cent W.

**DISCUSSION** There is no marked difference in distribution of tungsten with the variation in the compound ingested, i.e., the soluble sodium tungstate is

TABLE 1  
Distribution of tungsten

Diet	A		B		C		D		E		F		G	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
No. of Rats	4	4	2	2	2	2	2	2	2	2	2	2	2	2
Milligrams per cent of W														
Bone	0	0	11	8	11	9	9	10	10	10	14	18	11	18
Spleen	0	0	3	12	7	12	2	9	2	6	7	14	8	9
Skin	0	0	Tr	Tr	Tr	Tr	Tr	10	Tr	2	Tr	Tr	2	3

distributed approximately to the same extent as the less soluble metal. The mechanism of absorption of the metal is not evident but it is likely that some compound is formed by oxidation of the metal in the diet or after it reaches the gastro-intestinal tract, and then this product is absorbed in the intestine.

In order to remove any trace of oxide from the metal, Diet G was prepared from metallic tungsten which had been treated as described in the footnote. This purification produced no significant difference in distribution. Although Diet G contained no trace of oxide at the time the diet was prepared, it is still probable that oxidation occurred later.

The chief sites of deposition of tungsten are bone and spleen with somewhat smaller quantities in skin, kidney, and liver. Despite careful scrubbing and washing of the skin, it is possible that the tungsten found in the skin is due to contamination by the diet rather than the result of cutaneous deposition. This factor could not be definitely eliminated in these feeding experiments. Without exception, tungsten was found in the liver but the concentration in each case was less than 10 milligram per cent. Tungsten deposition thus differs from heavy

metal deposition in general for in the latter the storage is chiefly in the liver and to a less extent in the spleen, kidney, and bone (4)

Blood, lung, testis, and muscle in some instances showed storage of tungsten in traces (less than 1.0 milligram per cent) while in other instances no trace was found. The occasional presence of tungsten in lung and muscle appears to be independent of the presence of tungsten in the blood at the time of analysis, for in the two cases where blood showed no trace of the metal, both lung and muscle did show traces. The tungsten doubtless had been deposited in the two tissues at an earlier date and was no longer circulating in the blood.

Brain, heart, and uterus were free of tungsten except in a single instance in each organ. In the brain, it is possible that in removing this organ that a small spicule of bone was collected with the nervous tissue, but this is unlikely. There is no obvious explanation for the single positive finding in heart and uterus.

In no instance was there any trace of tungsten found in the animals kept on the control diet.

#### SUMMARY

In rats kept for 100 days on diets in which tungsten had been incorporated as either the metal, tungstic oxide, sodium tungstate, or ammonium paratungstate, the chief sites of deposition were bone and spleen with smaller quantities in the skin, kidney, and liver. The possibility that the finding of tungsten in the skin is an artefact is discussed.

Traces of the element were found in some instances in blood, lung, muscle and testis. In only a single instance was tungsten found in the brain, heart, or uterus.

In no case was tungsten found in any of the tissues of the control animals.

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EFFECT OF NH<sub>4</sub>Cl ON THE LIFE-MAINTAINING ACTION  
OF DESOXYCORTICOSTERONE ACETATE IN  
ADRENALECTOMIZED RATS

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It has recently been shown that the toxic manifestations of desoxycorticosterone acetate (D C A) overdosage can be prevented in experimental animals by the administration of ammonium chloride. High doses of D C A produce nephrosclerosis, cardiac lesions and periarteritis nodosa in rats kept on tap water. Administration of a 1% sodium chloride solution instead of water aggravates these toxic manifestations of the corticoid hormone, while 1% ammonium chloride prevents them (1). Systematic investigations concerning the effect of various electrolytes on D C A overdosage symptoms indicate that sodium bicarbonate shares with sodium chloride the ability to increase sensitivity to these damaging effects of the hormone, while the action of calcium chloride resembles that of ammonium chloride inasmuch as it protects against D C A intoxication (2). Contrary to earlier reports (3), we were unable to demonstrate any significant protection in animals receiving potassium chloride.

These observations revealed the possibility of counteracting some of the unwelcome manifestations of D C A by treatment with certain electrolytes. In view of the fact that overdosage symptoms frequently interfere with the clinical use of D C A, especially in patients suffering from Addison's disease, it appeared of practical importance to study the mechanism by which electrolytes protect against the toxic effect of this steroid. In this connection, the most important question was whether a substance such as ammonium chloride, completely inactivates the hormone or whether it merely antagonizes its toxic side effects. We therefore decided to determine the life-maintaining potency of D C A in adrenalectomized rats receiving tap water or ammonium chloride solution, respectively.

Immature male hooded rats, having an initial body weight of 40-55 g (average 46.5 g) were used for all the experiments of this series. Half of the animals received a 1% ammonium chloride solution *ad lib* instead of drinking water, while the other half was given tap water. On the 4th day both adrenals were removed through lumbar incisions in all animals of this series, then the rats were subdivided into 8 groups, as follows. Groups I, III, IV and V received tap water to drink, while Groups II, VI, VII and VIII were given a 1% ammonium chloride solution *ad lib*. Since adrenalectomized animals are very sensitive to any change in diet, these drinking fluids were given not only during the experiment, but also 3 days prior to adrenalectomy. Groups I and II acted as controls and received no D C A. Groups III and VI were given 50 $\gamma$ , Groups IV and VII 70 $\gamma$ , and Groups V and VIII 100 $\gamma$  of D C A. in 0.1 cc of peanut oil subcutaneously once

daily. Table 1 summarizes our results. A few rats died during the first 24 hours after the operation, apparently due to operative shock or hemorrhage, while another few proved to have small adrenal remnants when examined at autopsy. These animals are not listed in the table.

Perusal of table 1 indicates that the animals drinking ammonium chloride do not gain weight as well as the controls receiving water. This is in agreement with our previous observations on intact animals and indeed it is hardly surprising that animals kept on a 1% ammonium chloride solution are not in as good a condition as those which are allowed to drink water. The growth inhibition is manifest in the controls (Group II) as well as in the various D.C.A. treated

TABLE I

*Effect of NH<sub>4</sub>Cl on life-maintaining action of desoxycorticosterone acetate in adrenalectomized rats*

Group	I	II	III	IV	V	VI	VII	VIII
Treatment	H <sub>2</sub> O	NH <sub>4</sub> Cl	D.C.A. (50%) H <sub>2</sub> O	D.C.A. (70%) H <sub>2</sub> O	D.C.A. (100%) H <sub>2</sub> O	D.C.A. (50%) NH <sub>4</sub> Cl	D.C.A. (70%) NH <sub>4</sub> Cl	D.C.A. (100%) NH <sub>4</sub> Cl
No. of rats in group	32	31	30	22	10	28	21	10
Av. initial body weight in g	46	47	46	47	46	46	47	46
Av. final body weight in g	53	44	62	63	61	55	52	57
Deaths								
2nd day	3	4	1	2	0	1	1	0
3rd day	2	3	0	0	0	2	2	0
4th day	9	12	0	0	0	3	0	0
5th day	5	9	0	0	0	0	0	0
6th day	13	3	0	0	0	6	0	0
Total (in %)	100	100	33	9	0	42.8	14.3	0

The figures in this column represent the percentage of the animals, in each group which died during the entire 6 day period.

groups (Groups VI-VIII). Accordingly the total mortality rate is higher among the ammonium chloride-treated animals receiving threshold doses of 50-70% of D.C.A. (Groups VI and VII) than in the corresponding groups receiving the same amount of D.C.A. but given water (Groups III and IV). However 100% per day which is not much above the threshold life-maintaining dose is perfectly adequate to maintain adrenalectomized rats kept either on water or on ammonium chloride solution. It appears that ammonium chloride does not markedly counteract the life maintaining potency of D.C.A. since the survival rate of adrenalectomized animals kept on ammonium chloride and given 100% of this steroid is better than that of adrenalectomized controls given 50-70% of D.C.A., while receiving tap water. The inability of ammonium chloride to

annul the life-maintaining action even of a few gamma of D C.A. is noteworthy in view of the fact that in our earlier experiments—referred to above—the same concentration of ammonium chloride sufficed to counteract the toxic effects normally resulting from the daily administration of several milligrams of this steroid

These observations suggest that perhaps administration of ammonium chloride may permit the use of D C.A. in man with comparatively little danger of producing overdosage phenomena

#### SUMMARY AND CONCLUSIONS

The life-maintaining action of desoxycorticosterone acetate (D C.A.) is not significantly inhibited by the ingestion of ammonium chloride, although previous experiments showed that its toxic manifestations may be completely prevented by this electrolyte. These observations are noteworthy because they suggest a fundamental difference in the mechanism through which D C.A. exerts its life-maintaining and toxic effects respectively.

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# THE EFFECT OF CURARE AND CURARE-LIKE SUBSTANCES ON THE CENTRAL NERVOUS SYSTEM<sup>1</sup>

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Effects of curare on the central nervous system of cold and warm blooded animals have repeatedly been reported. The majority of these observations referred to changes in the activity of the central nervous system such as dampening of artificially produced stimulation, or the enhancement of the narcotic effects of other drugs. A direct effect on the central nervous system was demonstrated in experiments in which curare abolished the cortical potentials in frogs independently of its paralyzing action on myoneural junctions (Feitelson and Pick 1942). Since these experiments were carried out with crude extracts of curare which undoubtedly carried extraneous material, it was desirable to reinvestigate the effects on the central nervous system with a pure alkaloid of curare recently obtained in crystalline form (Wintersteiner and Dutcher, 1943). Furthermore it was our intention to extend these studies to other substances with a curare-like action on the myoneural junction. Accordingly d-tubocurarine chloride, dihydro- $\beta$ -erythroidine HCl quinine ethochloride, nicotine (base) and thiamine HCl were used in these experiments. In an attempt to analyze the effects of the curarizing drugs, experiments were also carried out with prostigmine acetyl choline, monoiodoacetic acid and sodium cyanide. All these substances which are readily soluble in water were used as neutralized solutions in 0.6% sodium chloride.

Gerard and his collaborators (Labet and Gerard 1938 1939 and 1941, Gerard and Libet, 1940) carried out the first studies on the electrical potentials of the isolated brain of the frog and especially of the lobi olfactori. They obtained specific reactions of these organs to various drugs which were applied locally. Since, obviously the frog's brain *in situ* with its natural blood supply affords more physiological conditions than the isolated brain our studies were carried out mostly on the brain potentials of pithed frogs, sometimes also on normal frogs paralyzed by curare-like drugs. Pithing of the frog abolished all reflexes of the spinal cord including the swallowing reflex which otherwise may interfere with the E.E.G. In our experiments only ocular movements sometimes interfered with the E.E.G. The reactions of the E.E.G. obtained in this manner were

<sup>1</sup> The senior author (E. P. Pick) wishes to express his thanks to the Ella Sachs Plotz Foundation and to the Daxian Foundation for Medical Research for financial aid to Dr. O. Wintersteiner of the Squibb Institute for Medical Research for a gift of d-tubocurarine, Dr. Oliver Kamm of Parke-Davis and Company for a gift of quinine-ethochloride and Dr. L. Pirk of Hoffmann LaRoche Inc for a gift of prostigmine.

The investigation was begun in collaboration with Dr. S. Feitelson of the Mount Sinai Hospital who had to discontinue the work because of other duties.

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not in every respect identical with those obtained by Gerard and co-workers on the isolated frog brain. The reactions of the frog brain differed from those of warm blooded animals most markedly in that convulsant drugs like strychnine and picrotoxin injected intralymphatically in doses of 4 mgm per kgm failed to have any noticeable effect on the electric potentials of the frog brain. It may be recalled that Kolm and Pick (1920) in perfusion experiments on frogs failed to demonstrate the existence of a vasomotor center which responded to strychnine or picrotoxin. Furthermore, Bremer, Dow and Moruzzi (1939) reported that these drugs are likewise ineffective in stimulating the brain of reptiles. Caffeine with which Gerard and Libet (1940) obtained specific effects on the isolated lobes olfactores and hemispheres of the frog, caused in our experiments only a fleeting period of increased electrical activity for 1 or 2 minutes immediately following the injection of 20 to 400 mgm of caffeine sodium benzoate per kgm. The E.E.G. of the frog is also less sensitive to lack of oxygen (Sugar and Gerard, 1939), to the action of acid and alkali and to changes in the pH than the electrical cortical potentials of mammals (Libet and Gerard, 1939). Hypo- and hyperglycemia appear to have little if any influence on the E.E.G. of the living frog, a finding in agreement with the statement of Gerard and Libet (1940) that the isolated frog brain is completely indifferent to the presence or absence of glucose in the surrounding salt solution. On the other hand, numerous experiments on the frog's brain *in situ* have shown that the E.E.G. is extremely sensitive to the action of drugs paralyzing the central nervous system.

**METHODS** The experiments were carried out on frogs of 30 to 50 gram body weight in a manner similar to that employed in previous experiments (Feitelberg and Pick, 1942). Heagland (1940) electrodes were inserted (1) at the frontal end of the left hemisphere, (2) at the occipital end of the right hemisphere, and (3) at the region of the lobes optici of the left hemisphere. Records were made by a Grass three channel ink writing oscillograph. In order to conserve space, the record of only one channel is presented in the illustrations. Prior to the insertion of the electrodes, the frogs were pithed. In some experiments the paralyzing effect on the striated muscles was studied in the intact animals, after complete recovery, the frogs were pithed and the effects of the same drug on the E.E.G. were studied in the usual manner. Furthermore, E.E.G. were taken of intact (not pithed) animals subjected to paralyzing doses of various drugs. All substances were administered by injection into the thoracic lymph sac. The effect of every drug was studied on at least 12 frogs.

**RESULTS** 1. *d-tubocurarine* Crystalline *d*-tubocurarine chloride (Wintersteiner and Dutcher, 1943) caused reversible paralysis in doses of 3 mgm per kgm frog. It proved to have the same effects on the E.E.G. of frogs as the crude curare preparation in previous experiments (Feitelberg and Pick, 1942). Small amounts of *d*-tubocurarine (3 mgm per kgm) which paralyze the skeletal muscles, usually failed to exert any influence on the E.E.G. Doses of 4 and 7.5 mgm per kgm, however, abolished the electrical potentials for periods which lasted considerably longer than the peripheral effect of this substance (fig. 1, A and B). Frogs recovering from the effects on the striated muscle were able to jump in an apparently normal fashion, although their E.E.G. failed to show any regular electrical potentials at this time. Thus, the central and the peripheral

action of d tubocurarine chloride appear to be independent of each other as indicated previously in experiments with crude curare (Fentelberg and Pick, 1942) A distinction between the central and peripheral action was also found when prostigmine (0.1 mgm. per kgm.) was injected into the curarized frog. Although prostigmine hastened to restore the motility of the animals it failed to

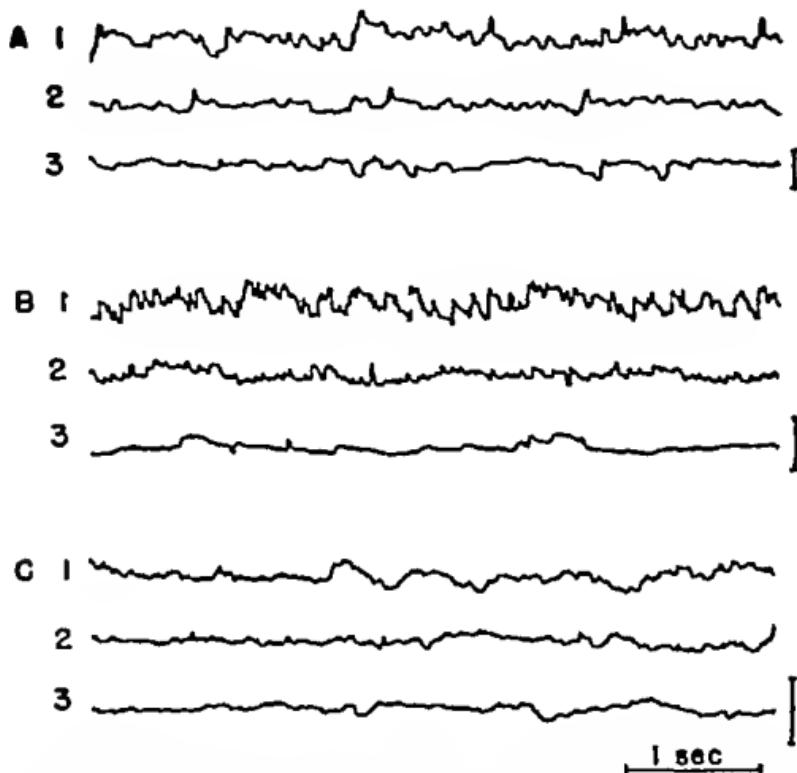


FIG. 1. EFFECT OF d TUBOCURARINE ON THE BRAIN POTENTIALS OF THE FROG

A Frog 50 grams. Record 1—10 minutes, Record 2—18 minutes Record 3—41 minutes after injection of 4 mgm. per kgm. d tubocurarine. The frog was completely paralyzed before Record 1 was taken. Amplitude 9 mm. 50 micro-volts; channel 1 speed 3 cm. per 1 second.

B Same frog, next day. Pithed after recovery. Record 1 before Record 2—4 minutes, Record 3—18 minutes after injection of 4 mgm. per kgm. d tubocurarine. Amplitude 12 mm. 50 micro-volts; channel 1 speed 3 cm. per 1 second.

C Frog 35 grams. Record 1—15 minutes after injection of 5 mgm. per kgm. d tubocurarine. Frog is completely paralyzed. Record 2—10 minutes later 1 minute after injection of 0.2 mgm. per kgm. prostigmine. Record 3—17 minutes after prostigmine. The frog was able to jump. Brain waves remained abolished for another hour. Amplitude 15 mm. 20 micro-volts; channel 2 speed 8 cm. per 1 second.

restore the brain potentials to their normal amplitude (fig. 1 C). Likewise, strychnine (5 mgm. per kgm.) and picrotoxin (5 mgm. per kgm.) were ineffective in restoring the electrical activity of the brain of curarized frogs.

2. *dihydro-beta-erythroidine* The curare-like action of alkaloids obtained from various species of the genus *Erythrina* has recently been described (Unna and

Greshin, 1944) Among these alkaloids dihydro-beta-erythroidine was one of the most potent, blocking the myoneural junction in frogs in doses of 0.5 mgm per kgm. In analogy to its effect upon curare, prostigmine was found to antagonize the action of dihydro-beta-erythroidine in cold and warm blooded animals (Unna, Kniazuk and Greshin, 1944). In our experiments dihydro-beta-erythroidine was given in doses from 0.5 to 2 mgm per kgm. The smallest dose often failed to influence the EEG, although it was sufficient to paralyze the skeletal muscles. Larger doses (1 and 2 mgm per kgm) had an effect on the EEG similar to curare. A few minutes after the injection the amplitude and frequency of the potentials decreased gradually and all brain potentials had disappeared.

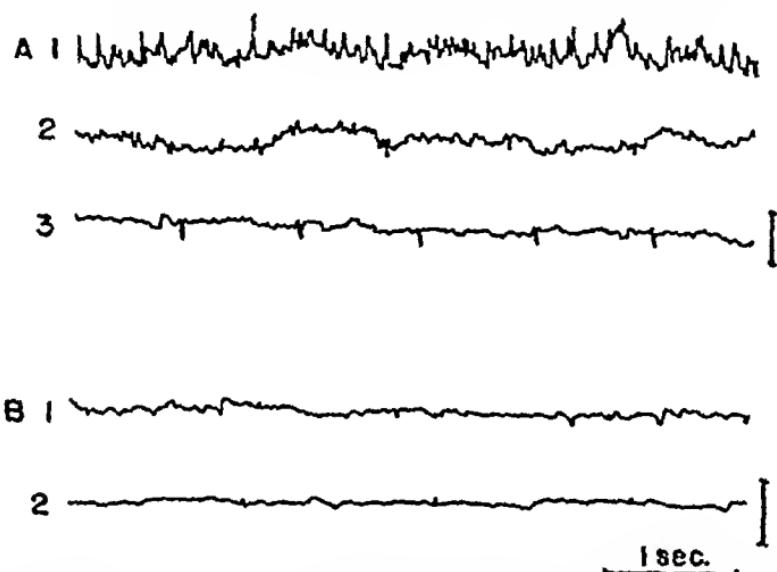


FIG. 2. EFFECT OF DIHYDRO-BETA ERYTHROIDINE ON THE BRAIN POTENTIALS OF THE FROG

A. Frog 45 gram, pithed. Record 1—before, Record 2—10 minutes, Record 3—30 minutes after injection of 2 mgm /kgm dihydro beta-erythroidine. Amplitude 12 mm 20 microvolts, channel 2, speed 3 cm per 1 second.

B. Frog 40 gram. Record 1—10 minutes after 2 mgm /kgm dihydro beta-erythroidine (complete muscle paralysis), Record 2—17 minutes after 0.1 mgm /kgm prostigmine (motorility restored). Amplitude 15 mm 20 microvolts, channel 3, speed 3 cm per 1 second.

after 20 to 30 minutes (fig 2). On the other hand, complete block of the myoneural junction following the same doses of dihydro-beta-erythroidine became manifest within 6 to 9 minutes. A further differentiation between the central and peripheral action of this substance was shown by the effect of prostigmine. As in curarized frogs, prostigmine (0.1 to 0.2 mgm per kgm) accelerated the recovery from the effects of dihydro-beta-erythroidine upon the skeletal muscles, but failed to counteract the depression of the brain waves (fig 2, B). The interruption of the synaptic transmission in the brain by dihydro-beta-erythroidine persisted at a time when the peripheral transmission at the myoneural junction was restored either spontaneously or following the administration of prostigmine.

Since dihydro-beta-erythroidine produced the same effects on the E E G of the frog as d-tubocurarine it seemed of interest to learn whether one of these alkaloids would enhance the effects of the other. Therefore, a dose of d-tubocurarine (3 mgm. per kgm.) was injected which was so small as to show no influence on the E.E.G. One hour later, a small dose of dihydro-beta-erythroidine (0.5 mgm. per kgm.) known to be insufficient to cause an alteration of the brain waves in normal frogs was given. The amplitude and frequency of the potentials decreased within a few minutes. Thus, it appears that the two alkaloids mutually enhance each other in their effects upon the brain of the frog. In this connection it may be of interest to note that curare as well as dihydro-beta-erythroidine potentiate the effect of anesthetics on the heat production in certain parts of the

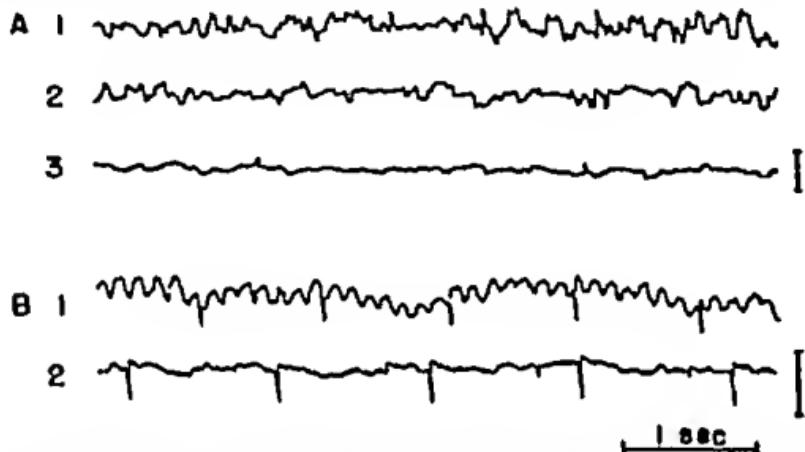


FIG. 3. EFFECT OF QUININE ETHOCHLORIDE ON THE BRAIN POTENTIALS OF THE FROG

A. Frog 52 grams pithed. Record 1—before. Record 2—2 minutes, Record 3—20 minutes after injection of 40 mgm./kgm. quinine ethochloride. Amplitude 9 mm. 50 micro-volts; channel 1 speed 3 cm. per 1 second

B. Frog 22 gram. Record 1—14 minutes after 40 mgm./kgm. quinine ethochloride (complete muscle paralysis). Record 2—20 minutes after the injection. Amplitude 16 mm. 50 micro-volts; channel 2 speed 3 cm. per 1 second

brain of cats. Both substances decrease, in a like manner, the heat production in the brain, independently of their peripheral action (Pick and Feitelberg, 1944).

3. Quinine ethochloride. The curare-like action of quinine methochloride has been described by Harvey (1940) and that of quinine ethochloride by Chase and Lehman (1942). In normal frogs this substance caused a reversible paralysis 20 minutes after the administration of 40 mgm. per kgm. In pithed frogs 40 mgm. per kgm. caused a gradual decrease in the amplitudes of the brain waves, and 15 to 30 minutes after the injection the brain potentials had almost disappeared (fig. 3). The depression of the electrical activity of the brain lasted for 1½ hours or longer. This compound prolonged the atrio-ventricular conduction time in the same manner as quinine as evidenced by the electrocardiogram obtained in the record of fig. 3. The minimum doses of quinine ethochloride effective in depressing the brain potentials and in blocking the neuromuscular junction were

identical, as was the duration of both the central and peripheral effects. Prostigmine failed to counteract the effect of quinine ethochloride on the brain potentials of frogs.

**4 Prostigmine and Acetylcholine** The observation that prostigmine failed to counteract the effect of any of the curarizing agents on the brain potentials prompted us to study the effect of prostigmine alone and in conjunction with atropine and acetylcholine on the EEG of pithed frogs. It was found previously (Pick and Feitelberg, 1944) that prostigmine failed to increase the heat production of the brain of cats. Only when the oxidative metabolism of the brain

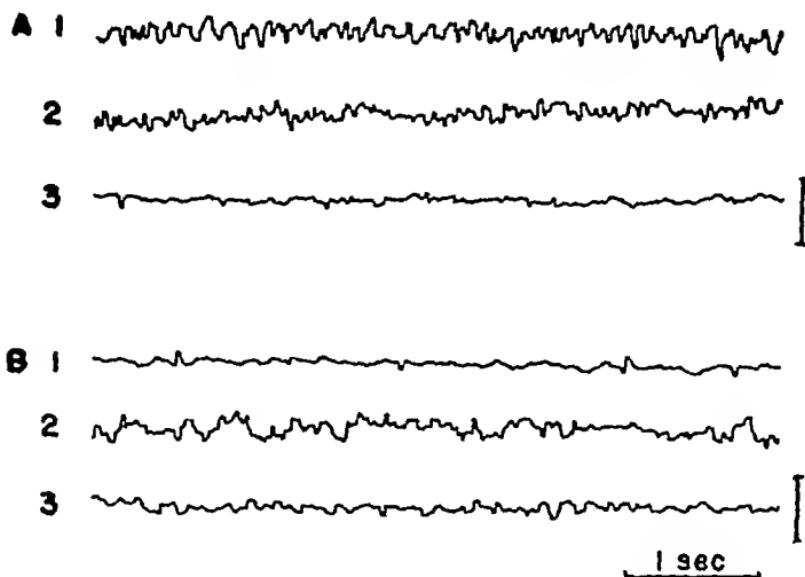


FIG. 4. EFFECT OF PROSTIGMINE AND ACETYLCHOLINE ON THE BRAIN POTENTIALS OF THE FROG

A. Frog 42 grams injected with 2.5 mgm per kgm atropine and pithed. Record 1—before, Record 2—1 minute, Record 3—7 minutes after injection of 0.12 mgm /kgm prostigmine. Amplitude 15 mm 50 micro volts, channel 1, speed 3 cm per 1 second.

B. Frog 42 grams 2.5 mgm /kgm atropine, pithed. Record 1—10 minutes after injection of 0.12 mgm prostigmine. Record 2—immediately following injection of 40 micrograms per kgm acetylcholine, Record 3—1 minute later. Amplitude 15 mm 50 micro volts, channel 2, speed 3 cm per 1 second.

was markedly increased as for instance following benzedrine, did prostigmine increase further the heat production. In frogs injections of 0.2 to 1.0 mgm per kgm prostigmine were followed by a decrease in the amplitude and frequency of the potentials (fig. 4, A). Administration of atropine did not influence the effects of a subsequent injection of prostigmine. The depression of the electrical activity of the brain contradicts the assumption of a stimulation of central synapses by prostigmine. Whether this depression is due to a direct effect upon the synapses or indirectly through inhibition of stimulatory reflexes remains to be seen. Our findings appear to be comparable to the initial effects observed by Chatfield and Dempsey (1941) who on applying prostigmine directly to the cerebral cortex

of cats recorded a transient depression of spontaneous activity both at the area of application and elsewhere.

Acetylcholine (0.06 to 0.2 mgm. per kgm.) increased for a period of 30 seconds the electrical activity of the brain in frogs which had received atropine and prostigmine prior to the injection of acetylcholine (fig. 4, B). The short phase of stimulation was followed by a long lasting depression of the electrical activity of the brain. Chatfield and Dempsey (1941) also observed an initial increase in spontaneous activity followed by rapid potentials of lower voltage when acetylcholine was applied to the cortex of cats which had received prostigmine.

*5. Nicotine sodium monoiodo acetate and sodium cyanide.* It has been shown (Libet and Gerard, 1938, Gerard and Libet, 1940) that the topical application of a 0.5% solution of nicotine to the exposed lobi olfactori of a freshly amputated frog head abolished the wink reflexes elicited by touching the eye or nostril, abolished visual action potentials evoked by shining light in the eye, and prevented the changes in olfactory bulb potentials otherwise produced by stimulation of the olfactory nerves. Similarly, in cats blocking of reflexes from the thalamus, spinal cord and the brain stem were observed following the intravenous injection of large doses of nicotine (2.5 mgm. per kgm.). These observations were interpreted as blockade of synapses in the central nervous system by nicotine.

The injection of nicotine in doses of 3 to 15 mgm. per kgm. in pithed frogs caused an immediate acceleration of the brain waves from 5 or 6 to 10 or 15 per second (fig. 5 A). This phase probably due to a stimulation of central sympathetic synapses lasted for about 10 minutes and was followed by a gradual decrease in the frequency of the waves. Fifteen to twenty minutes later, no electrical potentials could be recorded although the electrocardiogram remained unaltered. These findings differ from those of Gerard and Libet (1940) who observed on the isolated frog brain an increased amplitude of the potentials but unaltered or decreased frequency of the waves following nicotine. It seems possible that this phase of 'parasympathetic' stimulation of the lobi olfactori may have been masked in our experiments by the 'sympathetic' stimulation of the hemispheres of the frog.

In view of the fact that in the presence of nicotine brain tissue is able to oxidise glucose but unable to metabolise lactic and pyruvic acids (Baker and Hinwich 1938) the effect of monoiodoacetic acid on the E.E.G. was studied. Monoiodoacetic acid inhibits the enzymatic hydrolysis of carbohydrates in the tissues and thus decreases the formation of lactic acid (Lundsgaard 1929). The intralym phatic injection of sodium monoiodoacetate even in very large doses (100 mgm. per kgm.) caused, in contrast to nicotine, no immediate alteration of the brain potentials. After 5 to 10 minutes the amplitude of the potentials began to decrease slowly after about 30 minutes, even with maximum amplification, only faint potentials could be recorded. The slow disappearance of the electrical activity following monoiodoacetic acid during this period is probably indicative of the low metabolic rate of the brain tissue. During this phase of monoiodoacetic acid poisoning the heart action and circulation remained unaffected. The frog usually succumbed to 100 mgm. per kgm. of sodium monoiodoacetate after 2

hours. An inhibition of lactic acid formation appears not to be responsible for the suppression of the electrical potentials, since the administration of lithium lactate to the frogs treated with monoiiodoacetic acid failed to restore the electrical potentials.

A combination of monoiiodoacetic acid and nicotine shortened the phase of increased electrical activity which followed nicotine alone and led to a more rapid decrease and disappearance of the electrical potentials than either substance alone. Gerard and Libet (1940) also found that the combination of these two

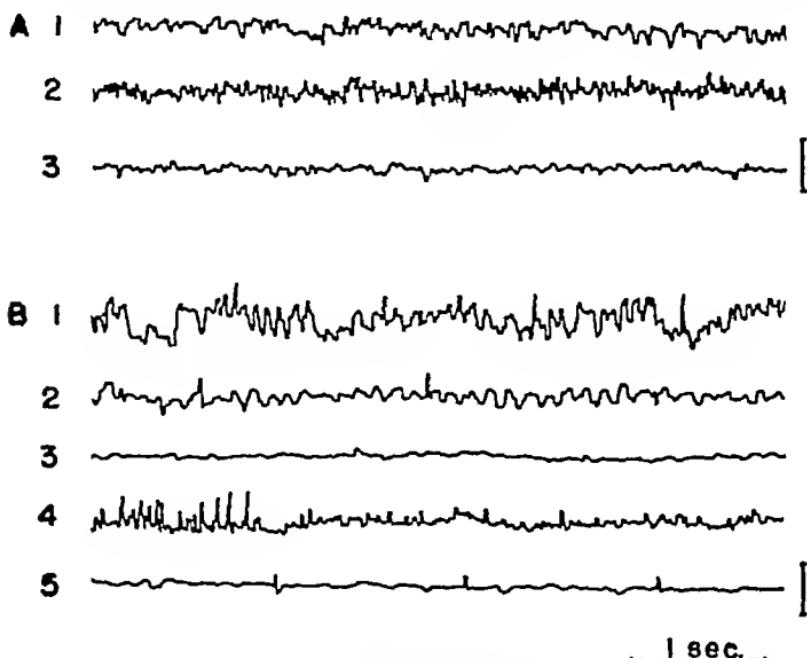


FIG. 5. EFFECT OF NICOTINE AND OF SODIUM MONOIODOACETATE ON THE BRAIN POTENTIALS OF THE FROG

A. Frog 43 gram, pithed. Record 1—before, Record 2—immediately after, Record 3—8 minutes after injection of 10 mgm/kgm nicotine. Amplitude 12 mm 20 micro-volts, channel 3, speed 3 cm per 1 second.

B. Frog 35 grams, pithed. Record 1—before, Record 2—2 minutes after, Record 3—8 minutes after injection of 80 mgm/kgm sodium monoiiodoacetate. Record 4—immediately after 10 mgm/kgm nicotine (2 minutes after Record 3), Record 5—11 minutes later. Amplitude 12 mm 20 micro-volts, channel 2, speed 3 cm per 1 second.

substances suppressed the stimulatory phase of nicotine, although monoiiodoacetic acid alone (0.004 molar) in their experiments caused a transient increase in the frequency of the waves from 6 to 15 per second together with a considerable decrease in amplitude.

The effect of sodium cyanide was compared to that of monoiiodoacetic acid. In the isolated frog brain, sodium cyanide (0.003 to 0.005 molar) causes a decrease in the amplitude and increase in the frequency of the electrical potentials, which may persist for hours (Gerard and Libet, 1940). In our experiments, the injection of 50 mgm per kgm of sodium cyanide was followed by a transient phase of

increased frequency of the brain potentials. The amplitude of the potentials and later also the frequency of the waves decreased gradually. Upon repeating the injection of sodium cyanide the electrical activity of the brain ceased completely although the heart was still beating regularly. These findings in agreement with the results of Gerard and Libet, bear evidence of the remarkable resistance of the frog brain against anoxia.

6 *Thiamine* Curare-like effects of excessive doses of thiamine have been reported by Demole (1938). While rats following the intravenous injection of 0.2 gram per kgm. succumbed to respiratory paralysis frogs receiving 1.5 gram per kgm. showed a reversible paralysis during which the muscle remained responsive to direct stimulation. In confirmation of Demole's observations we found that injections of 1.0 or 1.2 gram per kgm. of thiamine paralyzed frogs for about 24 hours and that prostigmine accelerated the recovery. These curanizing effects

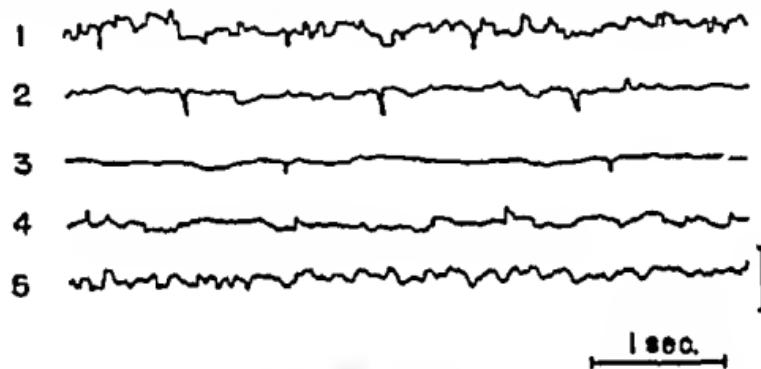


FIG. 6 EFFECT OF THIAMINE ON THE BRAIN POTENTIALS OF THE FROG

Frog 35 gram, pithed. Record 1—before Record 2—2 minutes Record 3—44 minutes Record 4—120 minutes and Record 5—150 minutes after the injection of 6 mgm./kgm. thiamine. Amplitude 15 mm. 50 micro volts channel 3 speed 3 cm. per 1 second

of thiamine and the observations made on the inhibition of synaptic transmission in the plexus of Auerbach (Unna and Pick 1944) prompted us to study the effect of thiamine upon the E.E.G. of the frog. In studies on the E.E.G. of pigeons it had been shown that thiamine failed to influence the electrical activity of the normal pigeon's brain, the administration of thiamine to avitaminotic pigeons however produced a reversion of the altered potentials toward normal (Tokaji and Gerard 1939). In man, the oral and intravenous administration of 100 mgm. of thiamine daily over a period of 6 to 9 weeks failed to cause an alteration of the E.E.G. of schizophrenic patients (Rubin, 1939).

In pithed frogs, thiamine in doses of 3 to 10 mgm. per kgm. caused a transient increase in the frequency of the brain waves. After several minutes the frequency and the amplitude of the potentials decreased considerably. In the majority of the experiments the electrical activity was completely suppressed for several hours (fig. 6). Prostigmine was ineffective in counteracting the effects of thiamine on the E.E.G.

The inhibition of the electrical activity of the brain apparently paralleled the

effect of thiamine on nervous transmission in sympathetic ganglia (Unna and Pick, 1944). Whether the effects of thiamine observed in the E.E.G. were accompanied by an alteration in carbohydrate metabolism has not been studied. However, the depression or complete suppression of the brain potentials following the administration of thiamine would indicate a severe derangement of the normal metabolic processes in the brain tissue. It is well known that thiamine may inhibit cholinesterase (Glick and Antopol, 1939) and phosphatase (Glick, 1942), two enzymes playing an important role in metabolic processes associated with the transmission of nerve impulses. Since acetylcholine in large amounts paralyzes the synapses of the central nervous system (Bonvallet and Minz, 1938, Schweitzer and Wright, 1939, Chatfield and Dempsey, 1941) it appears possible that the inhibition of the electrical activity of the frog brain obtained by thiamine is due to an accumulation of excess acetylcholine.

**DISCUSSION** The experiments herein presented demonstrate that substances which block the myoneural junction (*d*-tubocurarine, dihydro-beta-erythroidine, quinine ethochloride, nicotine and thiamine) are also able to influence the electrical activity of the frog brain. The effects observed in the E.E.G. of pithed frogs consisted in a decrease of the frequency as well as a diminution of the amplitudes of the potentials which could be entirely suppressed by larger doses. With curare and erythroidine the doses effective in decreasing the electrical activity of the brain were slightly larger than those which block the transmission at the myoneural junctions. On the other hand, thiamine suppressed the electrical potentials of the brain in doses representing only 1/20 or 1/50 of those effective on the myoneural junction. However, these amounts of thiamine appear still to be considerably larger than the minute quantities necessary for an adequate functioning of carbohydrate metabolism.

The effect upon the E.E.G. of all substances studied, with the exception of quinine ethochloride, differed from that on the myoneural junction in that the inhibition of the electrical activity of the brain was of considerably longer duration and persisted after the function of the myoneural junctions was restored. It appears likely that the changes observed in the E.E.G. indicate a blockade of the synapses of the central nervous system by these substances as indicated previously by experiments with nicotine (Libet and Gerard, 1938). The inhibition of the electrical activity of the brain following monooiodoacetic acid was to be expected from studies on the inhibition of the carbohydrate metabolism in the frog's spinal cord and the loss of spinal reflexes following the administration of this substance (Leedebur, 1932, Holmes, 1933). However, lithium lactate, in contrast to its partial effectiveness in restoring the functions of the spinal cord poisoned with monooiodoacetic acid failed to counteract the effects of monooiodoacetic acid on the E.E.G. Sodium cyanide, in a manner similar to that of monooiodoacetic acid, inhibited and almost completely suppressed the electrical activity of the frog brain.

The effects observed in the E.E.G. differ from the peripheral effects of these substances further in that the effects on the central nervous system are not reversed by prostigmine. Prostigmine alone depressed the electrical activity of the

frog brain. Schweitzer, Stedman and Wright (1939) have demonstrated that prostigmine depressed spinal reflexes in cats and may even abolish strychnine convulsions by its inhibitory effect upon the spinal cord. Recent clinical observations (Kabat and Knapp, 1944) made on cases of spasticity treated with prostigmine have led to the assumption that the therapeutic effect may be attributed to an inhibition of proprioceptive reflexes in the spinal cord by prostigmine.

The increased use of curare and of substances with a curare-like action in the therapy of spasticity has renewed the interest in the action which these substances have on the central nervous system. West (1937) reported that the relief from spasticity is not associated with the true curare action at the neuromuscular junction and concluded that the beneficial effect in certain patients is due to a central effect ("lissive action"). Our findings with d-tubocurarine, dihydro-beta-erythroidine and quinine ethochloride emphasize that consideration should be given to the effects on the central nervous system which these substances may exert in the treatment of various neurologic conditions.

More recently curare has been used as an adjuvant in relaxing abdominal muscles during inhalation anesthesia, particularly by cyclopropane (Griffith and Johnson 1942, Cullen 1943). It has been found useful especially for patients who are resistant to the anesthetic agent (Baird and Adams 1944). However the application of curare in patients as well as in dogs has shown (Cullen 1948) that this drug alone failed to produce a satisfactory relaxation of the peripheral muscle and that the combination with scopolamine and the anesthetic agent was necessary to produce and to maintain a satisfactory relaxation. The findings presented in this paper in particular the potentiation by curare and dihydro-beta-erythroidine of the effect of anesthetics on the heat production in certain parts of the brain, direct attention to the possibility that the effects of curarizing drugs on the central nervous system contribute a significant share in the therapeutic effects obtained by clinical investigators. In view of the inherent danger in the administration of curarizing drugs, it should be pointed out that prostigmine which counteracts the action of these drugs on the neuromuscular junction itself suppresses the electrical activity of the brain. Consequently as would be expected prostigmine failed to reverse the depression of the electrical activity of the frog brain caused by curarizing drugs.

#### SUMMARY

1. Crystalline d-tubocurarine, dihydro-beta-erythroidine, quinine ethochloride, nicotine and thiamine inhibited and suppressed the electrical activity of the frog brain.

2. Prostigmine which itself depresses brain potentials, failed to influence the effect of these substances on the brain potentials although it counteracted their effects on the neuromuscular junction.

3. Prostigmine partially inhibited the electrical activity of the frog brain. A subsequent injection of acetylcholine suppressed the electrical activity completely.

4 Monooiodoacetic acid and sodium cyanide abolished the brain potentials of pithed frogs

5 The findings are interpreted as an inhibition of central synaptic transmission by curare and substances with a curare-like action

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# CHEMOTHERAPY AND ANTITOXIN THERAPY OF EXPERIMENTAL CL. WELCHII INFECTION IN MICE<sup>1</sup>

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In contrast to the great progress which the advent of the sulfonamides marked in the treatment of infections caused by various pathogenic cocci the sulfonamide therapy of anaerobic wound infections has until recently proved of only limited value. Reports from the present war (1) indicate that local sulfonamide treatment of wounds even when preceded by surgical cleansing of the potentially infected area, cannot by itself be relied upon as a safe method for preventing the development and spread of gas gangrene. There are clinical reports (2 3) however, which suggest that a combination of local sulfonamide therapy with the early administration of antitoxin may be more effective than the use of either drug or serum alone. First clinical trials of penicillin in the treatment of gas gangrene (4, 5) have given encouraging results, though there are indications that the simultaneous administration of antitoxin may be indispensable.

In dealing with this problem one has to take into account that gas gangrene may be caused by several species of clostridia and that the frequent lack of an early differential diagnosis adds to the difficulties of comparing under war conditions the effectiveness of different treatments. It appeared desirable, therefore to assess in laboratory animals experimentally infected with definite species of pathogenic clostridia, the value of chemotherapy and of serum therapy as well as of the combination of these.

Experimental evidence has been presented by previous workers (6-9) which indicates that the combination of chemotherapy with antitoxin therapy may give better results than either drug or serum alone. The recent study by McIntosh and Selbie (9) includes experimental use of penicillin confirming its activity against *Cl. welchii* as reported by Hac and Hubert (11).

A new compound which appeared to be of particular interest for inclusion in our study is p-(ammo-methyl)-benzenesulfonamide. It was claimed by Domagk (12) and Klarer (14) to possess a marked activity against clostridia though Miller et al. (15) had previously described it as ineffective against streptococci. This sulfonamide, used in Germany under the name of 'Mariamil', was synthesized in our laboratory in 1943 with the assistance of John R. Policy. It was compared in regard to its activity against clostridia with other chemotherapeutic agents. Included among these were N<sup>1</sup> benzoyl sulfanilamide<sup>2</sup> (No. 191), a compound of low toxicity and marked activity against pneumococci (16 17),

<sup>1</sup> This investigation was supported by a grant in aid from the National Research Council in Ottawa (MP 29).

<sup>2</sup> Effectiveness of this sulfonamide in gas gangrene was found by Prof G B Reed (Queen's University Kingston) to whom the drug was submitted for trial in 1943.

as well as sulfathiazole previously described as giving some protection against experimental gas gangrene (10, 18-22).

The first part of this study, as presented here, deals with *Clostridium welchii* infections. According to recent reports (23) it appears that *C. welchii* still constitutes, as it did in the last war (22), the main causative agent of gas gangrene, though it is frequently found associated with other anaerobic and aerobic pathogens (24).

For our investigation the following general plan was followed. To determine, against *C. welchii* infections in mice the effectiveness (a) of various chemotherapeutic agents, including penicillin, (b) of antitoxin and (c) of the combination of chemotherapy with antitoxin therapy.

**METHODS** *Infection of mice* A strain of *C. welchii* (B P 6 K), as used by Dr. Edith Taylor for the production of perfringens toxin was found to have a satisfactory virulence for mice. Its virulence was maintained by weekly pigeon passage. For each mouse experiment a fresh 4 hour (37°C) subculture, grown in beef heart casein hydrolysate broth, was used. The centrifuged growth was washed and resuspended in beef broth. A broth dilution was prepared to contain approximately four million bacilli per 0.05 cc., using a hemocytometer for making a direct microscopic count (Armstrong and Rae, 1941 (20)). This suspension was further diluted immediately before use with an equal volume of a 5% calcium chloride solution. Of this mixture 0.1 cc. was used as a test dose for intramuscular injection into the median aspect of the right thigh. The presence of 2.5 mg calcium chloride in each test dose proved adequate to insure maximum virulence of the culture, though twice this amount was used for the initial experiments.

For each experiment a subculture dilution count, using semisolid nutrient agar tubes, was made which confirmed the presence in each test dose of 2 to 6 million viable clostridia (average of 3.5). For mice of 19-22 grams this dose corresponds to 5 to 10 m.l.d.'s.

The deaths were recorded up to the 10th day after infection. Some of the mice were kept under observation for an additional 10-14 days and were finally used for studying their resistance to a second infection with *C. welchii*.

*Administration of therapeutic agents* *Sulfonamides* For local injection into the infected area, the sulfonamides were given as a finely ground suspension (or solution) in 0.8% sodium chloride solution, containing 1% gelatine. For oral administration the drugs were suspended (or dissolved) in a 5% gum acacia solution and fed twice daily for three days by means of a stomach tube, the first dose being given one hour after infection. For all sulfonamides the dosage used lay far below the toxic level. The only exception was the intravenous administration of 10 mg Marfanil, as twice this dose proved to be toxic for mice. The intramuscular and oral administration of Marfanil (up to 50 and 200 mg respectively) were well tolerated.

*Penicillin* Penicillin as produced by the Banting Institute was used. Dilutions of the sodium salt were made up in 0.8% sodium chloride solution and injected intramuscularly in 0.1 cc. amounts at the site of infection. Similar solutions were used for intravenous injection.

*Antitoxin.* Polyvalent Gas Gangrene antitoxin was used as prepared for the Armed Forces by the Connaught Laboratories. It contained per cc 745 units of *Perfringens* antitoxin in addition to 370 units of *Septicæ* antitoxin and 745 units of *Oedematiens* antitoxin. The initial dose contained in 0.3 cc, was given intraperitoneally, one hour after infecting the mouse in the case of early treatment and four hours after infection in the case of late treatment. In either case the treatment was repeated subcutaneously once daily for three days.

*RESULTS.* (a) *Chemotherapy.* In determining the activity of various drugs in the early local treatment of *Cl. welchii* infections in mice it was found that of all sulfonamides tested Marfanil is by far the most active. As may be seen from table 1 0.5 mg Marfanil is as effective or more effective than 2 mg of the other sulfonamides including sodium sulfadiazine. The only chemotherapeutic agent which for local treatment compares favourably with Marfanil is penicillin. The dose of 2 mg Marfanil is equivalent to 50 units of penicillin saving the life of approximately 90% of the infected mice. Higher doses of Marfanil offer no advantage.

In view of the marked activity of Marfanil and of penicillin in local therapy it was of importance to find out how these two therapeutic agents compare when administered by other routes. In table 2, a comparison is recorded between local chemotherapy on one hand and intravenous or intramuscular therapy on the other. In the case of penicillin, a dose of 50 units represents an effective dose for early local treatment but this same dose is ineffective when given intramuscularly into the thigh of the non infected leg. However when the dose is increased fivefold to 250 units it becomes nearly as effective as the 50 units injected locally at the site of infection. A single intravenous injection of penicillin (250 units) is fairly effective when administered one hour after infection. When given as late as four hours after infection, it is without effect. The mice so treated all die in less than 24 hours after infection.

The Marfanil treatment (table 2) differs essentially from penicillin treatment in that its useful effectiveness is limited to local application. The early intramuscular injection into the non infected leg of 10 mg Marfanil corresponding to five times the locally effective dose does not give the mice a satisfactory protection. This equally applies to the early (one hour) intravenous administration of 10 mg Marfanil which saves the life of only 20% of the mice, in contrast to 85% in the case of penicillin.

In studying the effect of oral administration of sulfonamides (table 3) it was found that, against the intramuscular infection of mice with 5 to 10 m.l.d.'s of *Cl. welchii*, Marfanil is ineffective whereas sulfadiazine and especially sulfa thiazole (4 mg. twice daily) delay death noticeably leaving the occasional survivor.

That oral administration of sulfonamides may under more favourable conditions have a more beneficial effect is shown by an experiment (table 3) in which sulfathiazole sulfonamide 191 and Marfanil were fed in larger doses (10 mg. twice daily) to groups of 10 mice, infected intramuscularly with only 1 to 2 m.l.d.'s of *Cl. welchii*. Under this condition Marfanil and sulfonamide 191 af-

forded protection to 70% and 90% respectively, whereas with sulfathiazole all mice survived

TABLE 1  
*Local chemotherapy of *C. welchii* infection in mice*

SINGLE TREATMENT*	DOSE	NO OF TESTS	NO OF MICE	SURVIVORS (10TH DAY)
Sulfathiazole	1.0 mg	4	30	10
	2.0 mg	4	50	30
Sulfadiazine	1.0 mg	3	20	5
	2.0 mg	2	30	13
Sulfadiazine (Na-salt)	1.0 mg	2	10	30
	2.0 mg	2	20	35
Sulfamerazine†	1.0 mg	1	10	30
	2.0 mg	2	20	20
Sulfamethazine‡	1.0 mg	1	10	10
	2.0 mg	2	20	45
No 191 = N <sup>1</sup> Benzoysulfanilamide	1.0 mg	2	20	20
	2.0 mg	2	30	33
No 203 = p (Amino methyl) benzenesulphonamide hydrochloride = "Marfanil"	0.2 mg	2	20	10
	0.5 mg	3	40	40
	1.0 mg	5	40	82
	2.0 mg	2	30	93
	5.0 mg	2	20	80
Penicillin (Na-salt)	5 units	2	10	50
	10 units	1	15	40
	25 units	2	20	85
	50 units	6	60	88
Penicillin (4 hrs) §	25 units	1	20	0
Controls		16	158	0 6

\* Local intramuscular injection one hour after infection

† 2-sulfanilamido-4-methylpyrimidine described by Roblin et al (36) and Welch et al (37)

‡ 2-sulfanilamido-4,6-dimethylpyrimidine described by Macartney et al (38)

§ Local intramuscular injection four hours after infection

Grateful acknowledgement is made of the following supplies Sulfadiazine and its methyl derivative received from Dr R O Roblin, American Cyanamide Company, and sulfamethazine from Dr C M Scott of Imperial Chemical Ltd

*Local chemotherapy with drug mixtures* In view of the frequent presence in wounds of staphylococci and streptococci, the admixture to Marfanil of other

sulfonamides which are effective against pathogenic cocci, appears justified. Two different mixtures of sulfathiazole with Marfanil (1:1 and 7:3) were tested in mice (table 4). On a weight per weight basis, both mixtures are less effective than Marfanil alone, which result may be expected in view of the very much lower

TABLE 2

Chemotherapy of *Cl. welchii* infection in mice Comparison of penicillin with Marfanil

SINGLE TREATMENT	TIME AFTER INFECTION	DOSE	NO. OF TESTS	NO. OF MICE	SURVIVORS (10TH DAY)
Marfanil local	hr				%
Marfanil i.m.	1	2 mg	2	30	93
Marfanil i.v.	1	10 mg	1	10	20
Penicillin local	1	60 Units	2	20	100
Penicillin i.m.	1	50 Units	2	20	5
Penicillin i.m.	1	250 Units	2	20	70
Penicillin i.v.	1	250 Units	2	20	85
Penicillin i.v.	4	250 Units	1	20	0
Controls			5	50	0

Intramuscular injection into the thigh of the non-infected leg

TABLE 3

Oral sulfonamide therapy of *Cl. welchii* infection in mice

INTRAMUSCULAR IN- JECTION IN M.L.D.'S	TREATMENT	DOSE <sup>a</sup> PER OS	NO. OF TESTS	NO. OF MICE	SURVIVORS (10TH DAY)
5-10	Sulfathiazole	mg			%
	Sulfadiazine	4	3	30	23
	191	4	2	20	10
	203	4	1	10	0
	203	10	2	20	5
			1	10	0
Controls			3	30	0
1-2	Sulfathiazole	10	1	10	100
	191	10	1	10	70
	203	10	1	10	90
Controls			1	10	0

\* Administered twice daily for 3 days First treatment one hour after infection

activity of sulfathiazole in the local treatment of *Cl. welchii* infections (see table 1). The results indicate that the activity of such mixtures increases with their Marfanil content.

In order to test for a possible existence of synergism between penicillin and

sulfonamide treatment, two groups of mice were compared (table 4), the mice of both groups being injected locally with 10 units of penicillin one hour after infection, but one group receiving in addition 2 mg of sulfathiazole suspended in the penicillin solution. Although the number of mice used was small, the results do indicate that for the early treatment of *Cl. welchii* infections such a combination offers no advantage over the use of penicillin alone.

As a third possibility of combining for local treatment different types of chemotherapeutic agents, *sulfathiazole-proflavine* mixtures were tested. Such a mixture (100:1) has been tried previously in the treatment of experimental *Cl. welchii*

TABLE 4

*Local chemotherapy of *Cl. welchii* infection in mice Testing of drug mixtures*

SIMPLE TREATMENT			NO. OF MICE	SURVIVORS (10TH DAY)
Drug	Dose	Time after infection		
Marfanil, alone	1 mg	hr	10	90
Sulfathiazole, 1 part + Marfanil, 1 part	{ 1 mg 2 mg	1	20	75
Sulfathiazole, 7 parts + Marfanil, 3 parts	{ 2 mg 10 mg 10 mg	1	10	40
		4	10	90
			15	0
Penicillin, alone	10 units	1	15	40
Sulfathiazole* + Penicillin	{ 2 mg 10 units	1	16	25
Controls			30	0

\* Suspension in penicillin solution.

infections (9). In two experiments (not recorded in table form) groups of 20 ( $2 \times 10$ ) mice received the following local chemotherapy one hour after infection:

A 2 mg of sulfathiazole,

B 2 mg of a sulfathiazole-proflavine mixture (100:1),

C 2 mg of a sulfathiazole-proflavine mixture (9:1) and

D 0.2 mg of proflavine.

The percentage survivals (10th day) were for group A—15%, group B—35%, group C—50% and for group D—25%, indicating that the sulfathiazole-proflavine mixtures were somewhat more effective than either the sulfathiazole or the proflavine alone. Even the best results (group C), however, do not measure up to the 80–100% protection obtained regularly when using 2 mg of Marfanil for local treatment (table 1). Judging by percentage survivals, the sulfathiazole-proflavine mixture B (100:1) is about equivalent to the sulfathiazole Marfanil (7:3) mixture (see table 4).

*Gangrenous lesions.* Although some of the described forms of chemotherapy are effective in saving the lives of a large percentage of the infected mice, they do

not as a rule prevent the development of the local anaerobic infection which may take the following forms

- (a) edema of the thigh or of the whole leg frequently followed by
- (b) a state of typical gangrene, which depending on its extent may lead to the loss of the whole limb or of the lower part of the leg. In some cases the infection of the thigh does not lead to this advanced gangrenous condition but persists as
- (c) a local subacute infection forming either an open wound or a deep scar

Mice which owing to chemotherapy, survive the third day, do as a rule live for several weeks irrespective of whatever local lesions they develop. The control mice receiving no treatment die within less than 48 hours usually within 24 hours and frequently show local swelling but no fully developed gangrene.

The injection into the thigh of normal mice of 2.5-5 mg. calcium chloride contained in 0.1 cc. of broth, produces no toxic symptoms apart from some local

TABLE 5  
Antitoxin therapy of *Cl. welchii* infection in mice

TREATMENT		NO. OF TESTS	NO. OF MICE	SURVIVORS (10TH DAY)
DOSAGE, International Units	Time after infection			
2	hr	3	30	20
5	1	1	20	85
25	1	2	20	95
250	1	2	20	80
25	4	4	36	60
Controls		9	90	0

\* First antitoxin treatment given intraperitoneally. Dose repeated subcutaneously once daily for three days after infection

swelling and a temporary inactivation of the leg without leaving a visible lesion. Higher doses (10-20 mg.) are toxic, frequently killing mice within 24-48 hours.

(b) *Antitoxin therapy* The effect of early administration of *perfringens* antitoxin was studied comparing different dosages (table 5). A dose of 25 International Units corresponding to 50-80,000 units for a human adult injected intraperitoneally one hour after infection repeating the dose subcutaneously once daily for three days, protects 95% of the mice. However as in the case of chemotherapy, the antitoxin treatment is incapable of preventing the development of the local infection in a large percentage of the animals. Even when using overwhelming doses of antitoxin (250 International Units daily) such local lesions still develop.

For the late treatment of *Cl. welchii* infections antitoxin therapy proves to be definitely superior to penicillin treatment. When the latter is given four hours after infection either locally (table 1) or intravenously (table 2) it is ineffective, while antitoxin (table 5) is still capable of saving the lives of 60% of the mice.

(c) *Combination of chemotherapy with antitoxin therapy* For the study of the combined effect of drug and serum treatment of *Clostridium welchii* infections, such forms of chemotherapy had to be selected which by themselves give a relatively high degree of protection when a sufficiently high dosage is used. However, in order to detect a possible synergism between chemotherapy and antitoxin therapy, the dosages used for the combination experiments had to be chosen so that neither the drug nor the serum alone would save more than 50% of the infected animals. This consideration applied both to the local chemotherapy with Marfanil as well as to the oral administration of sulfathiazole. As may be seen from the results obtained in using such combination experiments as recorded in table 6A, there is definite evidence of synergism between serum and drug treatment, both in the case of local Marfanil therapy as well as oral administration of sulfathiazole. In both cases the percentage survivals as obtained with the combined treatments are significantly higher than those obtained with either the drug or the antitoxin alone.

A convenient method for recording results of such experiments, permitting a comparison of various treatments, is shown in figure 1, where the percentage survivals are plotted against time. Here the combination of antitoxin treatment with the local administration of Marfanil (table 6A) is taken as an example. It shows a characteristic feature of all *C. welchii* experiments, which is that more than nine-tenths of all deaths occur before the end of the third day, a point already emphasized when describing the local infections (see above).

Of special interest is the finding that the combination of early sulfonamide therapy with serum treatment, in addition to saving the lives of a considerable number of mice, has the advantage over the use of either drug or serum alone, of reducing the occurrence of gangrenous lesions at the site of infection (table 6A, col 7).

This particular advantage of the combined treatment becomes an outstanding feature, when large dosages are used (table 6B). Here, both the serum as well as the drug alone are capable of saving the lives of 80% of the mice and the only advantage of the "sero-chemotherapy" finds expression in a reduction in the number of severe local infections observed.

Essentially the same results were obtained when comparing the combination of local penicillin therapy with large doses of antitoxin (table 6B). The best results as regards percentage survival and prevention of local lesions, were obtained in combining repeated local penicillin therapy with antitoxin therapy, using for the latter a dosage of 25 International Units per 20 gram mouse (table 6C). Only four of 30 mice so treated showed definite local lesions.

Of great practical importance is the question whether there is synergism between penicillin and antitoxin therapy in the case of late (4 hr) treatment of *C. welchii* infections. As shown previously (tables 1 and 2) penicillin alone is, under these conditions, without effect, while antitoxin alone is capable of saving the life of some of the infected animals (table 5). The experiments recorded in table 6D indicate that the combination of late intramuscular injection of large

TABLE 6

Effect of combining sulfonamide or penicillin therapy with antitoxin therapy in *Cl. welchii* infection in mice

TREATMENT	DOSE/ROUTE	START OF TREATMENT AFTER INFECTION	NO. OF TREAT.	NO. OF MICE	SURVIVAL RATE (10TH DAY)	PERCENT SHOWING LOCAL LESIONS
A	Marfanil 0.5 mg. local	1 hr	1	3	80	43
	Antitoxin 2 I.U., i.p.	1 hr	1	3	80	100
	Marfanil + Antitoxin 0.5 mg. local 2 I.U. i.p.*	1 hr	1	3	80	56
	Sulfathiazole 4 mg per os twice daily for 3 days	1 hr	1	3	80	100
	Sulfathiazole + Antitoxin as above 2 I.U., i.p.*	1 hr	1	3	80	70
B	Marfanil 5 mg. local	1 hr	1	2	20	80
	Antitoxin 250 I.U. i.p.	1 hr	1	2	20	80
	Marfanil + Antitoxin 5 mg. local 250 I.U. i.p.*	1 hr	1	2	20	95
	Penicillin 50 Units local	1 hr	1	2	20	75
	Penicillin + Antitoxin 50 Units local 250 I.U., i.p.*	1 hr	1	2	20	95
C	Penicillin 25 Units local†	1 hr	1	2	30	83
	Antitoxin 25 I.U., i.p.	1 hr	1	2	20	85
	Penicillin + Antitoxin 25 Units local† 25 I.U. i.p.*	1 hr	1	2	30	100
D	Penicillin 250 Units i.m.‡	4 hr	1	30	0	
	Antitoxin 25 I.U. i.p.*	4 hr	2	35	72	86
	Penicillin + Antitoxin 250 Units i.m.‡ 25 I.U. i.p.	4 hr	2	35	92	37
	Controls		0	89	0	

Local refers to intramuscular injection at site of infection—unless otherwise indicated a single injection was made

\* Dose repeated subcutaneously, once daily for 3 days

† Dose repeated twice daily for 3 days

‡ Dose repeated once 18 hr after infection treatment given in non infected leg

doses of penicillin (250 units) with antitoxin therapy protects more animals than does antitoxin alone. The lower incidence of severe local lesions (table 6D, col

7), observed in using the combined treatment, further adds to the evidence presented above, which demonstrates the value of "sero-chemotherapy" in *Cl. welchii* infection.

(d) *Recovering Cl. welchii from the infected mice* Of 195 control mice, which had not received treatment, and which died within less than 48 hours after infection, the heart blood was tested for the presence of *Cl. welchii* by inoculating it by means of a capillary pipette into semi-solid nutrient agar. Only in the case of 61% of these mice did *Cl. welchii* grow (37°), showing the typical lanceolate colonies. This particular strain of *Cl. welchii* (B P 6 K) proved, therefore, to be less invasive than two strains of *Cl. septicum*, which we used, in which instance

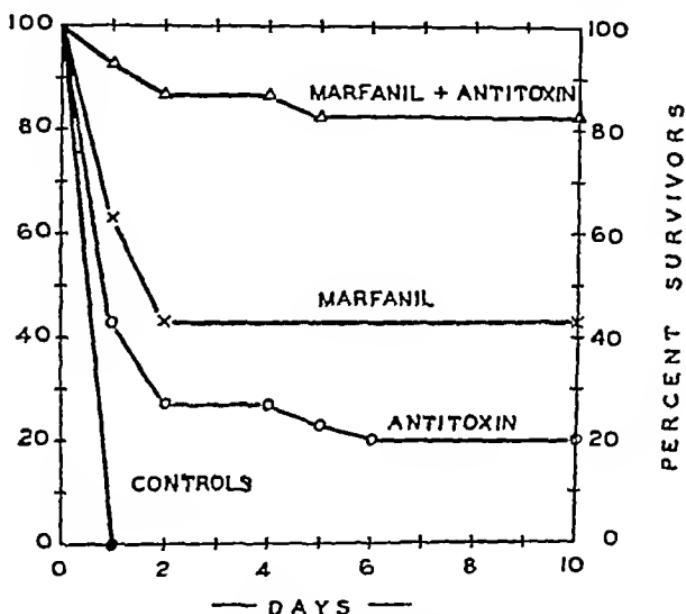


FIG. 1 TREATMENT OF *CL. WELCHII* INFECTION IN MICE WITH COMBINATION OF LOCAL MARFANIL THERAPY WITH SYSTEMIC ADMINISTRATION OF ANTITOXIN—(SEE TABLE 6, A)

more than 90% of the untreated mice showed after death the presence of *Cl. septicum* in the blood.

Thirty mice, which despite receiving treatment, developed gangrenous lesions, were killed between the 13th and 21st day after infection. Of each mouse a piece of thigh muscle was placed into Robertson's medium (37°C). In every instance *Cl. welchii* developed, irrespective of what form of treatment the mouse had received.

(e) *Resistance to a second infection with Cl. welchii* In order to test whether the surviving mice had developed some immunity against *Cl. welchii*, two hundred and thirty-nine survivors were reinfected with 5-10 ml d's of *Cl. welchii* culture three weeks after the first infection. Five days later the results showed the following percentage survivors: 51% of 148 treated with sulfonamides (sulfa-

thiazole sulfadiazine, sulfamethazine, Marfanil N' benzoyl sulfanilamide) and 21% of 91 treated with penicillin. From these figures it appears that the mice which had undergone sulfonamide treatment developed more frequently a resistance to a second infection than those which had received penicillin.

In a study, to be reported elsewhere of the absorption of sulfonamides and of penicillin after intramuscular injection in mice, it was shown that the above recorded resistance to a second infection is not caused by the possible presence in the muscle of remaining traces of the drugs with which the mice had been treated.

**DISCUSSION** A marked superiority of Marfanil over other sulfonamides in the treatment of anaerobic infections, as claimed by Domagk (12) is confirmed by our experiments, as far as *Cl. welchii* is concerned. However, in agreement with a recent report by Hamre et al. (29), we found this activity essentially limited to the local application of this drug while Domagk claims Marfanil to be effective both in oral and local administration. In our experience (table 3) only in the case of a very light infection, is the oral administration of Marfanil effective. This may explain the inability of some German workers (30) to confirm Domagk's finding. In using Marfanil subcutaneously in mice against experimental gas gangrene, Schreus et al. (31) found the drug ineffective while later experiments (32) indicated that the drug is active when applied at the site of infection.

In contrast to Hamre et al. (29) we found the local activity of sulfadiazine and its sodium salt consistently lower than that of Marfanil. Owing to its marked "in vivo" activity against *Cl. welchii*, as well as against *Cl. septicum* as shown by Domagk (12) and in our still unpublished work Marfanil is of practical interest. This is further emphasized by reports by Domagk (13) and by Schreus (33) that in contrast to other sulfonamides Marfanil does not lose its activity in the presence of p-aminobenzoic acid. As p-aminobenzoic acid proves to be an important metabolite synthesized by many pathogenic bacteria (34) a recent report (35) of favourable results obtained with Marfanil in the local treatment of general wound infections, deserves attention. Anticlostridial activity similar to that shown by Marfanil has recently been described for three other derivatives of p-toluene sulfonic acid (39).

Although our experiments show in agreement with those of previous workers (10, 18, 21, 25, 26) that local sulfonamide therapy may prevent the fatal outcome of the anaerobic infection they further indicate that frequently the local infection is not eliminated but may develop into a severe local lesion. In some cases true gangrene develops. The survival percentages or the average time of survival, often used for evaluating chemotherapeutic activity do therefore present an incomplete picture of the limited effectiveness of the treatment. The same consideration applies to antitoxin and penicillin therapy which do not prevent the later development of the local infection in some of the survivors.

It has been shown previously (10, 20, 27, 28) that the necrotic action of calcium chloride on muscle tissue prepares a favourable milieu for the development of the anaerobic infection. Although the injection of calcium chloride (2.5-5 mg.) alone does not produce any permanent visible lesion it may be argued that its use

in combination with the anaerobic infection, may give an unrealistic and unfavourable picture of the limited effectiveness of chemotherapy and of antitoxin therapy. Inducing such severe local infections has, however, the advantage of clearly revealing differences in the effectiveness of various treatments. While it is evident that, under the conditions of our experiments (table 6, A-D), neither chemotherapy nor antitoxin therapy alone eliminate the local infection, the combination of the two treatments markedly reduces the incidence of severe local lesions, proving thereby the superiority of the combined treatment.

The finding that even large doses of antitoxin (table 6B) alone do not prevent the later development of gangrenous lesions, indicates the absence of any marked anti-bacterial activity of the antitoxic serum.

The demonstrated development in mice of some degree of immunity to *Clostridium welchii*, may explain why some of the treated mice, after surviving for the first few days, live on for a longer period, despite the persistence of extensive local lesions.

The synergism between sulfonamide or penicillin therapy on the one hand and antitoxin therapy on the other, is likely due to the complementary nature of the two types of treatment, the former being essentially anti-bacterial, counteracting the invasiveness of the clostridial infection, while the latter acts by neutralizing the toxin formed. The experimental demonstration of such a synergism has practical implications. It not only emphasizes the necessity of early intravenous administration of antitoxin combined with chemotherapy, but it speaks in favour of abandoning the very large sulfathiazole doses, frequently used, in favour of a more moderate dosage, justifying a recent trend in the treatment of gas gangrene as recorded by MacLennan (2). A moderate dosage has the advantage of greater safety avoiding the possible ill-effects of critically high sulfathiazole blood levels in advanced cases of gas gangrene.

Although Marfanil is more active against *Clostridium welchii* than sulfonamides of the sulfanilamide type, its limited effectiveness against some strains of *Clostridium oedematum* and *Clostridium septicum* represents a definite limitation, which in our experience does not equally apply to penicillin. However, a point in favour of Marfanil is its relative chemical stability.

As long as sulfonamides are used in the early local treatment of war wounds, the use of Marfanil, in conjunction with a sulfonamide, more specifically active against pathogenic cocci, appears justified.

Our experiments indicate that irrespective of what form of chemotherapy may be chosen, the administration of antitoxin at the earliest possible moment is imperative.

#### CONCLUSIONS

1. In the early local treatment of *Clostridium welchii* infection in mice p-(amino-methyl)-benzenesulfonamide (Marfanil) proved to be considerably more effective than any other sulfonamide tested, including sodium sulfadiazine. Against the particular strain (B P 6 K.) of *Clostridium welchii*, Marfanil was approximately four times as effective as sulfathiazole. There was no marked difference between

sulfathiazole sulfamethazine sulfamerazine, N<sup>1</sup> benzoyl sulfanilamide and sodium sulfadiazine, while free sulfadiazine was somewhat less effective.

2 For the early local treatment, 2 mg. Marfanil proved to be equivalent to approximately 50 units of penicillin (corresponding to 0.03 mg. crystalline sodium salt). On a weight per weight basis, and under the conditions of our experiment penicillin was therefore about 60 times more effective against *Cl. welchi* (B.P. 6 K.) than Marfanil. The latter has, however, the advantage over penicillin of greater chemical stability.

3 Oral administration of Marfanil proved effective only in the case of light infection. When administered intravenously in subtoxic doses (max. 10 mg. per 20 g. mouse) it gave little protection.

4 For the early local treatment a mixture of sulfathiazole with Marfanil (7:3) showed approximately the same activity against *Cl. welchi* as a sulfathiazole proflavine (100:1) mixture. Although sulfathiazole Marfanil mixtures are less effective against *Cl. welchi* than Marfanil alone their possible advantage in the treatment of mixed infections warrants investigation.

5 Early sulfonamide therapy—in the form of local Marfanil treatment or oral administration of sulfathiazole—when combined with antitoxin treatment, saved more lives than either drug or antitoxin treatment alone, in addition to reducing the incidence of local gangrenous lesions.

6 Similarly a marked synergism exists between early local penicillin therapy and antitoxin therapy.

7 Under the conditions of our experiments, the antitoxic serum was found to be the only therapeutic agent now available which in the case of late (four hour) treatment of *Cl. welchi* infections is capable of saving the life of some of the infected mice. Its effectiveness is markedly enhanced however by simultaneous intramuscular administration of large doses of penicillin.

8 Some of the mice which survived the infection owing to early chemotherapy proved resistant to a second infection with 5-10 m.l.d.'s of *Cl. welchi*. This finding indicates the development in the course of the infection of some degree of immunity against *Cl. welchi*.

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## NOTE ON THE IN VITRO INACTIVATION OF MORPHINE BY LIVER

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In a recent paper (1) it was concluded that morphine is oxidized by rat liver *in vitro*. The evidence for this consisted in the fact that boiling in acid for 30 minutes did not have any effect on the amount of morphine recovered after incubation with liver slices. It has now been shown that if the solution is autoclaved at 20 pounds instead of boiled morphine is quantitatively recovered i.e. conjugation not oxidation accounts for the disappearance of morphine. This is true for all the animals studied as shown in table 1.

TABLE 1

Conjugation of 1.0 mg morphine HCl by 500 mg (wet weight) of liver slices

The slices were suspended in 4.0 cc of Krebs bicarbonate solution in an atmosphere of 95% oxygen and 5% carbon dioxide for 3 hours at 37°. The morphine was estimated with the silico molybdate acid reagent. Autoclaved and unautoclaved controls were used as standards.

ANIMAL	MORPHINE RECOVERED	
	BEFORE AUTOCLAVING	AFTER AUTOCLAVING
Rat	mg	mg
Guinea pig	0.40	1.04
Cat	0.60	1.05
Dog	0.62	1.00
	0.45	1.04

The properties of the reaction previously ascribed to the oxidation of morphine apply to the conjugation. Further facts concerning the reaction as it occurs in dog liver are as follows. The livers of five day old puppies conjugate the drug. When 0.5 mg is added 68% is conjugated in 3 hours, 1.0 mg 55%, 2.0 mg 37%. M/1500 moniodoacetic acid, M/500 sodium cyanide, and M/50 sodium fluoride inhibit the reaction completely and conjugation cannot be restored by the addition of either glucuronic or acetic acids. Conjugation takes place in the absence of sulfate ion. Tests indicate that morphine is conjugated with glucuronic acid but large scale experiments must be done to prove this.

### SUMMARY

Morphine is conjugated *not* oxidized when it is incubated with liver slices *in vitro*.

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In a recent paper (1) it was concluded that morphine is oxidized by rat liver *in vitro*. The evidence for this consisted in the fact that boiling in acid for 30 minutes did not have any effect on the amount of morphine recovered after incubation with liver slices. It has now been shown that if the solution is autoclaved at 20 pounds instead of boiled morphine is quantitatively recovered, i.e. conjugation not oxidation accounts for the disappearance of morphine. This is true for all the animals studied as shown in table 1.

TABLE 1

Conjugation of 1.0 mg morphine HCl by 200 mg (wet weight) of liver slices

The slices were suspended in 4.0 cc of Krebs bicarbonate solution in an atmosphere of 95% oxygen and 5% carbon dioxide for 3 hours at 37°. The morphine was estimated with the silico-molybdate reagent. Autoclaved and unautoclaved controls were used as standards.

ANIMAL	MORPHINE RECOVERED BEFORE HYDROLYSIS	MORPHINE RECOVERED AFTER HYDROLYSIS
	mg	mg
Rat	0.40	1.04
Guinea pig	0.60	1.05
Cat	0.52	1.00
Dog	0.45	1.04

The properties of the reaction previously ascribed to the oxidation of morphine apply to the conjugation. Further facts concerning the reaction as it occurs in dog liver are as follows. The livers of five day old puppies conjugate the drug. When 0.5 mg is added 68% is conjugated in 3 hours. 1.0 mg 55% 2.0 mg 37%. M/1500 monooctoacetic acid, M/500 sodium cyanide, and M/50 sodium fluoride inhibit the reaction completely and conjugation cannot be restored by the addition of either glucuronic or acetic acids. Conjugation takes place in the absence of sulfate ion. Tests indicate that morphine is conjugated with glucuronic acid but large scale experiments must be done to prove this.

### SUMMARY

Morphine is conjugated, not oxidized when it is incubated with liver slices *in vitro*.

### REFERENCE

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# THE ACTION OF SOME SPASMOlytic SUBSTANCES ON UTERINE MOTILITY<sup>1</sup>

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There is an apparent contradiction between the clinical usefulness of spasmolytic drugs in certain uterine disorders and their action on uterine motility in experimental animals. Clark and Shires (1) have demonstrated that the diethylamino-dimethylpropyl ester of tropic acid (Syntropan) causes contraction of the intact and excised uterus of various species and yet Syntropan has been successfully used in the treatment of primary dysmenorrhea (2, 3). Since uterine motility is greatly independent of the nerve supply, it was of interest to study the effect of drugs (4, 5, 6) which relax smooth muscle spasm by a mechanism not related to innervation ("musculotropic"). The substances tested were the diethylaminoethyl esters of dihydroanthracene-carboxylic acid (D), fluorene-carboxylic acid (Pavatrine), phenyl-tropic acid in comparison with the tropic acid esters of tropine (atropine), diethylaminoethanol and diethylamino-dimethylpropanol (Syntropan).

**EXPERIMENTAL.** *Animal Uterus.* The effect of these substances has been tested on the non-pregnant uterus "in situ" of nineteen urethanized rabbits (three ovariectomized) and nine cats (six decerebrate, two spinal, one intact under chloralose anesthesia). Most animals had been pretreated with estrone or stilbestrol. In most experiments uterine motility was recorded as by Trendelenburg (7). In some experiments the resistance to saline solution passing through one uterine horn was measured.

The responses obtained in cats were very inconstant, especially in the decerebrate preparations. The animals very often struggled when the injections were made which probably produced effects not due to the direct action of the drugs on the uterus.

The results of the rabbit experiments, however, were very uniform. There was no discernible difference in the effect of these substances in ovariectomized and intact animals. The responses could be always reproduced, at least qualitatively. Essentially, D caused relaxation, Syntropan caused contraction and the remaining compounds either had no effect or they increased motility, when used in larger doses (3 mgm/kg. - 5 mgm/kg.). A typical record is seen in fig 1.

Experiments on the isolated rabbit's uterus suspended in Locke's solution at 37°C yielded practically the same results. Six horns from intact rabbits and the same number of preparations from ovariectomized rabbits were used. All animals had received estrone or stilbestrol. Table I gives the results in summary form.

<sup>1</sup> This investigation was aided by a grant from G. D. Searle and Company, Chicago, Ill.

*Excised Human Uterus* The action of these substances was observed on seventeen strips of six human uteri. Eighty five injections were made. The strips were taken immediately after hysterectomy. It is essential that they are



FIG 1 RABBIT URETHANE ANESTHESIA

Injection through cannula into jugular vein. From the top down uterus *in situ* up-stroke—contraction, carotid arterial pressure signal of injection time in minutes. From left to right diethylaminoethyl fluorene-carboxylate 5 mgm per kg diethylaminoethyl dihydroanthracene-carboxylate 5 mgm per kg diethylamino-dimethylpropyl tropate 5 mgm per kg. Between injections fifteen and fifty two minutes.

TABLE I

	RA. R.F. UTERUS		EXCISED HUMAN UTERUS	
	Isolated	1 tact	Longit. dinal	Circula
Diethylaminoethyl-dihydroanthracene-carboxy late	—	—	—	—
Fluorene-carboxylate	+	+	—	—
-phenyl tropate	+	+	—	—
tropate		+	+	—
Diethylamino-di methylpropyl tropate	+	+	+	—
Atropine	0	0	+	—

— = relaxation + = contraction 0 = no effect

prepared as thin as possible to assure satisfactory spontaneous motility. The strips about one inch long were suspended in Ringer's solution at 37°C. Motility can be well preserved for as long as 48 hours if the specimens are stored in

oxygenated Ringer's solution at ice-box temperature. Since different drug responses from different segments of animal uterus have been observed (8, 9), both longitudinal and circular strips were used. The latter were prepared by cutting along the circumference of the lower end of the uterus at about the level of the internal os.

The concentrations in which the substances were used ranged from  $10^{-5}$  gms per cc to  $10^{-4}$  gms per cc. On longitudinal strips the tropic acid esters had either no effect or they caused a slight increase in tone and frequency of the rhyth-

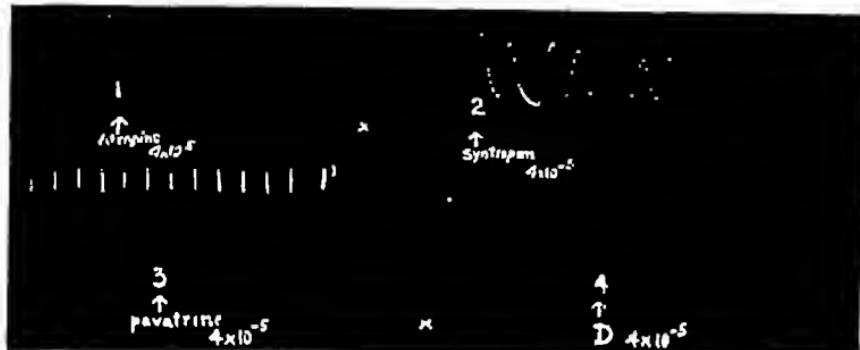


FIG 2 ISOLATED HUMAN UTERUS LONGITUDINAL STRIP

Upstroke = contraction, time in minutes.  $4 \times 10^{-5}$  grams per cc of 1 atropine sulfate, 2 diethylamino dimethylpropyl tropate, 3 diethylaminoethyl fluorene carboxylate, 4 diethylaminoethyl-dihydroanthracene carboxylate



FIG 3 ISOLATED HUMAN UTERUS (SAME AS IN FIG 2), CIRCULAR STRIP

From the top down, uterine motility, upstroke = contraction, time in minutes. From left to right at arrows  $4 \times 10^{-5}$  grams per cc of, diethylamino dimethylpropyl tropate atropine sulfate

mic contractions, whereas D, Pavatrine and the phenyl-tropic acid ester produced relaxation, the potency decreasing in the order mentioned (fig 2).

The motility of circular strips, however, was greatly reduced or abolished by Syntropin and atropine (fig 3). D and Pavatrine had the same relaxing effect as on longitudinal preparations.

**DISCUSSION** Animal experimentation seems inadequate for the evaluation of the clinical usefulness of uterine spasmolytic agents. Atropine (10, 11), Syntropin (2, 3) and Pavatrine have been reported to bring relief in some cases of dysmenorrhea while the circular strips taken from excised human uterus and the belief (13) that the pain

in some cases of primary dysmenorrhea is due to contraction of the *cervix uteri*. In addition spasmolytic drugs were found useful in obstetrics to hasten the dilatation of the cervix (14 15 16).

A relationship between structure and spasmolytic action is apparent. The tropic acid derivatives cause relaxation of human circular strips but cause contraction of human longitudinal strips and rabbit uterus. These compounds are effective acetyl-choline antagonists but are comparatively ineffective against histamine or barium (4). With the compounds containing two benzene rings (phenyl tropic acid and fluorene derivatives) compounds more effective than the tropic acid derivatives against histamine and barium relaxation of the longitudinal human strips but not the rabbit strips is seen. When a powerful histamine-antagonizing action accompanies an acetyl choline antagonizing action (D) all uterine tissue human and rabbit is relaxed.

#### SUMMARY

The effect on uterine motility of the diethylaminoethyl esters of dihydro-anthracene-carboxylic acid, fluorene-carboxylic acid (Pavatrine), phenyl tropic acid and of the tropic acid esters of tropine (atropine), diethylaminoethanol and diethylamino-dimethylpropanol (Syntropan) has been studied.

Of these compounds, the dihydroanthracene derivative is the only one which reduces or abolishes motility of the isolated and intact rabbit's uterus and of longitudinal and circular strips taken from excised human uterus.

Pavatrine and the phenyl tropic acid ester abolish motility of both longitudinal and circular strips of the human uterus however they usually cause contraction of the rabbit's uterus.

The tropic acid esters have a spasmolytic effect only on circular strips of the human uterus.

On the basis of these findings it is suggested that the pain of primary dysmenorrhea may be due at least in some cases, to spasm of the *cervix uteri*.

The findings of this investigation make intelligible the contradictory results of animal experimentation and clinical investigation concerning the effectiveness of uterine spasmolytics.

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# THE ANTI-HISTAMINE ACTIVITY OF DIETHYLAMINOETHYL-DIHYDROANTHRACENE-CARBOXYLATE AND OTHER SUBSTANCES<sup>1</sup>

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Many investigators believe that the manifestations of anaphylactic shock and allergic conditions are caused by histamine. For this reason many attempts have been made to find an agent capable of antagonizing the action of histamine or of preventing the occurrence of anaphylactic shock by interfering with the antibody-antigen reaction. In 1932, Hill and Martin (1) discussed 165 substances which had been tested for their anti-anaphylactic action. None of them was very effective. More recently phenolic ethers have been studied by Fourneau and his coworkers (2) which have been claimed to possess a specific anti-histamine action (3). The best known of these ethers is thymoxyethyl diethylamine. Although fairly potent it was never applied clinically because of its marked toxic side-effects (4). Certain aminoacids have been claimed to have a specific anti-histamine activity. However, very high concentrations of these aminoacids are required to antagonize the histamine effect on smooth muscle (5).

In a previous paper (6) we have studied the antagonistic action of diethylaminoethyl-dihydroanthracene-carboxylate (D) against the effects of histamine on smooth muscle. We have extended our investigation to study the antagonistic action of D and other substances (diethylaminoethyl-fluorene-carboxylate (F), diethylaminoethyl-anthene carboxylate (X), aminophylline and epinephrine) against anaphylactic shock.

**EXPERIMENTAL** *Anti-anaphylactic action "in vivo"* One hundred and one guinea pigs with an average weight of 400 grams were sensitized by the intraperitoneal injection of 200 mgm egg white (10% solution). The shocking dose was administered 18 to 21 days later. In control experiments the fatal dose of egg-white on intracardial injection was determined. Two mgm of egg-white killed 90% of the control animals. The substances which have been tested for their protective action were injected subcutaneously 30 minutes previous to the egg white. Of the compounds tested, only D provides complete protection against the effects of one fatal dose of antigen, whereas X is less effective. F has only a slight anti-anaphylactic action (table 1). These findings are in fair agreement with the relative potency of these compounds in relaxing histamine induced spasm of the isolated guinea pig's ileum (6, 9).

D lends protection to a few animals even if higher doses of egg-white are used, although the surviving animals show signs of severe respiratory distress. The mechanism of D in preventing death from anaphylactic shock is not through

<sup>1</sup>This investigation was aided by a grant from G. D. Searle and Company, Chicago, Ill.

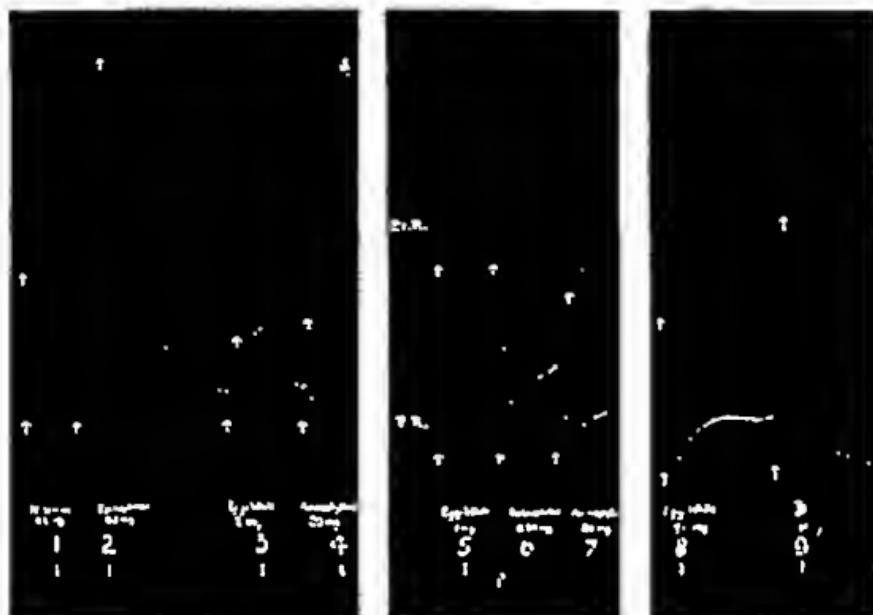


FIG. 1. PERFUSION OF ISOLATED LUNGS FROM THREE GUINEA PIGS SENSITIZED WITH EGG WHITE

From the top down bronchial resistance resistance in pulmonary circulation upstroke= increase in resistance signal of injection time in minutes. Injection through cannula into pulmonary artery 1—Histamine Phosphate 0.2 mgm. 2—Epinephrine 0.1 mgm. 3—Egg white 2 mgm. 4—Aminophylline 20 mgm. 5—Egg white 1 mgm. 6—Epinephrine 0.05 mgm. 7—Aminophylline 20 mgm. 8—Diethylaminoethyl-dihydroanthracene-carboxylate 1 mgm.

TABLE I  
Protection of guinea pigs against death from anaphylactic shock

NO. OF ANIMALS	PROTECTION % S.C. INJECTION OF	MG.M. EGG-WHITE INTRA CARDIALLY	DEAD	SURVIVED	% SURVIVAL
6	—	1	1	5	83
10	—	2	0	1	10
5	—	4	5	0	0
9	—	200	0	0	0
10	Diethylamino-ethyl-dihydro-anthra ceno-carboxylate 50 mgm./kg	2	0	10	100
10		4	5	4	40
15		200	11	4	27
10	Diethylamino-ethyl xanthene-carboxy late 50 mgm./kg	2	3	7	70
10	Diethylamino-ethyl fluorene-carboxy late 50 mgm./kg	2	7	3	30
8	Epinephrine 0.2 mgm./kg	2	5	3	37
8	Aminophylline 50 mgm./kg	2	5	3	37

interfering with the antibody-antigen reaction since those four animals which had been protected by D against the fatal effect of 200 mgm egg white did not show any symptoms when they received a subsequent injection of 200 mgm egg white two days later. The animals had been desensitized by the first shocking

egg-white injection, thus the protective effect of D is presumably due to its anti-histamine action on smooth muscle

*Anti-anaphylactic action "in vitro"* The anti-anaphylactic action of D, X, and F was tested on eleven uterine strips and of D, epinephrine and aminophylline on nine lungs taken from guinea pigs previously sensitized with egg-white. It is obvious that quantitative comparison cannot be obtained from these experiments since each experiment requires a new preparation. Nevertheless the findings are in agreement with those obtained from the "in vivo" experiments the order of spasmolytic potency being D > X > F if used in equal concentrations ( $3 \times 10^{-5}$  gms /cc.)

Thornton's method (7) has been used for studying the anti-anaphylactic effect of these substances on the isolated lung of sensitized guinea pigs. By this method both the resistance in the bronchioles and in the pulmonary circulation can be measured

Epinephrine, D, and aminophylline abolished bronchoconstriction induced by histamine or antigen injection. Epinephrine, however, augmented further the resistance in the pulmonary circulation from antigen injection, whereas the two other compounds diminished it (fig. 1). Aminophylline seems to have a greater influence on pulmonary than on bronchial resistance. It decreases the resistance in the pulmonary circulation after epinephrine, histamine and after antigen injection in preparations from sensitized animals. The effect of aminophylline may depend on the pre-existing resistance in the pulmonary circulation, since the resistance is increased by aminophylline in normal, unanesthetized dogs (8).

*Action of D on histamine-induced gastric secretion* If the anti-histamine action is a specific one (as atropine against acetyl-choline), then it should also inhibit histamine-induced gastric secretion

Three unanesthetized trained dogs were used for these experiments. The dogs received 20 mgm /kgm D subcutaneously, followed by the subcutaneous injection of 0.2 mgm /kgm histamine phosphate 30 minutes later. Three experiments were done in each dog and the same number of control experiments in which the injection of D was omitted. The dogs had been starved for 20 hours prior to the experiments. The stomach contents were aspirated through a stomach tube every 10 minutes for 60 minutes. The volume of the samples was measured, the free and total acid and the pH were determined. Sahli's reagent (equal parts of KI (48 per cent) and KIO<sub>3</sub> (8 per cent)) was used for the determination of the free acid. The total acid was determined by titration with N/100 NaOH with phenolphthalein as indicator. A Beckman pH-meter was used for the pH determinations. Table 2 shows the average values for each dog for pH, volume, and free acid as milligrams hydrochloric acid. Calculation of milligrams hydrochloric acid from the pH data agree with those obtained from titration of free acidity within the limits of error (error of pH measurements  $\pm 0.05$  pH). The reduction in volume of secretion and amount of hydrochloric acid are practically the same (average 32 per cent), which means that the acid concentration remains unchanged.

*Effect on capillary permeability* After the intravenous injection of trypan

blue in rabbits, wheals were produced by the intradermal administration of 0.2 cc. of 1:10,000 histamine phosphate solution and 0.2 cc. of a solution containing 1 per cent D in 1:10,000 histamine phosphate. The area infiltrated with histamine showed slight blue color whereas the color intensity was more marked at the site where the drug mixtures were injected.

TABLE 2  
Gastric secretion after 0.8 mg./kg. histamine phosphate subcutaneously

	MINUTES	VOLUME	FREE HCl <sup>a</sup>	pH <sup>b</sup>	20 MG./KG. D SUBCUTANEOUSLY 30 MINUTES PREVIOUS TO HISTAMINE		
					Volume	Free HCl	pH
Dog #1 female 15.1 kg	10	3.8	8.9	1.34	2.5	1.5	2.23
	20	14.3	50.8	1.12	7.2	23.8	1.17
	30	24.3	69.9	1.00	16.0	52.1	1.05
	40	22.7	84.0	1.05	13.0	47.5	1.03
	50	19.8	87.4	0.96	26.3	109.8	1.02
	60	21.5	93.0	0.96	13.7	50.6	0.96
Total		106.4	391.0		78.7	291.3	
Dog #2 male 12.2 kg	10	4.2	12.3	1.24	3.7	4.9	1.68
	20	12.6	48.1	1.08	4.7	10.8	1.82
	30	14.2	63.4	1.02	7.3	29.6	1.01
	40	14.0	65.3	0.95	9.7	40.6	1.04
	50	16.8	77.8	0.94	16.3	60.0	0.95
	60	12.9	58.7	0.96	14.8	64.8	0.94
Total		73.6	325.1		46.5	216.7	
Dog #3 male 18.7 kg	10	5.0	15.7	1.12	6.0	8.8	1.70
	20	19.6	83.1	0.98	21.7	81.2	1.05
	30	43.2	175.9	0.92	26.6	121.6	0.99
	40	44.5	225.2	0.91	26.7	132.0	0.94
	50	40.0	208.3	0.90	20.7	104.5	0.91
	60	42.0	210.0	0.86	26.7	133.5	0.91
Total		194.2	917.2		128.3	581.5	

Calculated from titration

<sup>b</sup> The pH was averaged by converting the pH readings into normality averaging the normality figures and reconvertng to pH

*Potency and toxicity of D* The potency was determined in the usual way (9) on the isolated guinea pig ileum against histamine induced spasm in comparison with epinephrine, thymoxyethylidethylamine,<sup>2</sup> and aminophylline. It is one fifth as active as epinephrine but three times stronger than thymoxyethylidethylamine and four thousand times more potent than aminophylline.

The toxicity of D and  $\lambda$  was determined in mice the results appearing in table 3.

<sup>a</sup> Generously supplied by the Abbott Laboratories North Chicago III

**DISCUSSION** The protective action of D is greater against histamine shock than it is against anaphylactic shock. It was 100 per cent effective against three fatal doses of histamine (6), but against only one fatal dose of antigen. In anaphylactic shock the histamine released is presumably closer to the effector organs of the smooth muscle cell. It recalls the antagonistic action of atropine on the intestine against injected acetyl-choline as compared with vagal stimulation.

TABLE 3

*Toxicity of diethylaminoethyl-dihydroanthracen-carboxylate and diethylaminoethyl xanthene carboxylate in mice*

	0.3	0.4	0.5	0.6	0.8	1.0	LD <sub>50</sub>
Oral							
D	gm/kg 3/10	gm/kg 4/10	gm/kg 4/7	gm/kg 6/7 0/4	gm/kg 3/9	gm/kg 4/8	gm/kg 0.46 1.0
X	0.4	0.6	0.8	1.2	1.5	1.8	LD <sub>50</sub>
Subcutaneous							
D	gm/kg 0/4	gm/kg 5/10	gm/kg 6/10	gm/kg 1/6	gm/kg 7/10	gm/kg 8/10	gm/kg 0.8 1.35
X	0.1	0.15	0.2	0.3			LD <sub>50</sub>
Intraperitoneal							
D	gm/kg 0/6	gm/kg 2/12	gm/kg 6/6 1/12	gm/kg 10/12			gm/kg 0.17 0.25
X	20	25	30	40	50		LD <sub>50</sub>
Intravenous							
D	mgm/kg 0/0	mgm/kg 0/5	mgm/kg 9/10	mgm/kg 6/6	mgm/kg 7/7		mgm/kg 27
X	1/7	8/9	6/6				22

$$\text{Mortality ratio} = \frac{\text{dead animals}}{\text{animals used}}$$

The moderately inhibitory effect of D on histamine-induced gastric secretion is not necessarily due to its anti histamine action. It may be attributed to the atropine-like action of this compound which has been previously demonstrated (6). It has been reported (10) that atropine decreases the volume but not the acid concentration of histamine-induced gastric secretion. Capillary permeability from histamine is not antagonized by D. The effect of D in increasing the color intensity of the wheals is presumably due to its marked vasodilator action (6). Thus the anti-histamine activity of this compound appears to be limited to

smooth muscle and even this does not seem to be very specific, as it has been shown (6) that it also relaxes smooth muscle spasm produced by other agents than histamine.

The marked effect of aminophylline in decreasing the resistance in the pulmonary circulation during an anaphylactic reaction may throw some light on the mechanism of its action in bronchial asthma. The bronchodilator effect of aminophylline (11) is so weak compared to epinephrine that it seems inadequate to explain the relief from aminophylline in many cases of acute asthmatic attacks when epinephrine has failed. It is quite possible that, in addition to the bronchoconstriction an increased resistance in the pulmonary circulation is an important factor in the pathogenesis of allergic asthma. We know that this is the case during anaphylactic shock in animals especially in the rabbit. If this factor is predominant then no relief can be expected from epinephrine which causes an increase in resistance. This suggests that the beneficial effect of aminophylline in bronchial asthma may be chiefly from a decrease in the resistance of the pulmonary circulation.

#### SUMMARY

Diethylaminoethyl-dihydroanthracene-carboxylate provides 100 per cent protection to sensitized guinea pigs against one fatal dose of antigen. Diethyl aminoethyl xanthene-carboxylate is 70 per cent effective. Diethylaminoethyl fluorene-carboxylate, aminophylline and epinephrine protect only 37 per cent of the animals.

The dihydroanthracene derivative reduces the volume but not the acid concentration of histamine-induced gastric secretion.

The dihydroanthracene derivative intensified the intradermal skin reaction produced by histamine.

The increase in the resistance of the pulmonary circulation of the isolated perfused guinea pig's lung caused by epinephrine, histamine or anaphylactic reaction is reduced by aminophylline and the dihydroanthracene derivative. The latter is more potent in its bronchodilator effect than aminophylline.

The possibility that the main mechanism of aminophylline in bronchial asthma is the decrease in the resistance of the pulmonary circulation, is suggested.

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## DIGITALIS AND CALCIUM

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Digitalis glucosides administered to isolated frog hearts filled with Ringer solution cause in time a decrease of the amplitude of contractions and eventually a standstill. The character of the decrease of the amplitudes and the time elapsing until the standstill depends on the dosage, with large doses the diastolic relaxation is diminished and after a short while a standstill in systolic contracture occurs. With minimal effective doses the systolic contractions very slowly decrease and eventually the heart stands still in diastole. Doses lying between these extremes produce effects lying between the aforementioned (Straub (1) and others). We know that calcium is involved in the systolic effect of digitalis (Clark (2), Loewi (3), and others). The present paper deals with the part calcium may play in the diastolic effect of threshold doses of digitalis.

The experiments were performed on isolated frog hearts (*rana pipiens*) attached to a Straub cannula. The Ringer solution had the following composition 0.65 grams of NaCl, 0.01 grams of KCl, 0.01 grams of CaCl<sub>2</sub>, 0.02 grams of NaHCO<sub>3</sub>, and water to make 100 cc solution. The digitalis preparations used were ouabain (Merck) and scillaren A (Sandoz). In adequate doses they affected the heart alike.

In order to find out whether calcium is involved in the diastolic action of threshold doses of digitalis, we first investigated the effect of calcium which was added at various intervals to hearts filled with Ringer solution and subjected to minimal effective doses of digitalis. Whereas raising of the calcium concentration to 0.02-0.04% and above this diminishes the diastolic relaxation of normal hearts filled with Ringer solution (0.01% CaCl<sub>2</sub>) after minimal effective digitalis doses this calcium effect is reversed, a short time after their application as a rule before any effect of this is visible a concentration of 0.02% calcium is ineffective. A concentration, however, of 0.04% CaCl<sub>2</sub> diminishes the systolic contraction, later on a concentration of 0.02% too displays a diastolic action and the decrease brought about by 0.04% CaCl<sub>2</sub> becomes bigger (fig 1). These results show that after the application of small doses of digitalis in Ringer solution additional calcium has a diastolic effect and that the calcium concentration producing this effect diminishes progressively. It is, therefore, most likely that in the gradual decrease of the heart contractions in Ringer solution following the application of small doses of digitalis, finally leading to a diastolic standstill, the calcium content of the Ringer solution is involved. This assumption is supported by the fact that after the diastolic state has been reached the application of a solution either free of or low in calcium concentration increases the contractions (fig 2). This increase, however, is only transitory. Eventually the heart stands still even in calcium poor solution.

A diastolic action of calcium, as observed here after the application of threshold doses of digitalis, so far has been unknown. How to explain it?

Since the systolic action of large doses of digitalis resembles the immediate action of high calcium concentrations and since small doses of digitalis act much more slowly than large ones, it seemed worth while to investigate whether large calcium concentrations if applied to a normal heart for a long time would also produce a diastolic action. This in fact is the case after having applied 0.04%



FIG 1 ♀ RINGER + 2 GAMMA PER ML QUABAIN    ♀ RINGER CONTAINING 0.02%  $\text{CaCl}_2$   
 ↑ RINGER CONTAINING 0.01%  $\text{CaCl}_2$



FIG 2 ♀ RINGER + 0.5 GAMMA PER ML QUABAIN    ♀ RINGER CONTAINING NO  $\text{CaCl}_2$  NOR  $\text{KCl}$   
 ↑ RINGER

$\text{CaCl}_2$  the usual immediate decrease of the diastolic relaxation in time gives way to a progressive decrease of the systolic contractions exactly like that following threshold doses of digitalis in Ringer solution but different in that in our experience high calcium concentrations even after hours do not lead to a standstill. If during the state of decreased systolic contractions one fills the heart with Ringer

solution of normal calcium content the heart recovers in that the systolic contractions reach their normal former size. The state of the heart however, in spite of this, has changed due to the long lasting action of the high calcium concentration. This becomes evident by the fact that refilling the seemingly recovered heart with Ringer containing 0.04%  $\text{CaCl}_2$  now does not produce any longer as in the normal heart a systolic but immediately a long lasting diastolic effect (fig 3). This by the way is to our knowledge the first case of an irreversible change of the state of the heart produced by cations.

**DISCUSSION** The object of these experiments was to find out whether calcium is involved in the diastolic action of small doses of digitalis as it is in the systolic action of large doses. From the outset this did not seem obvious since a diastolic action of calcium so far had been unknown. Our experiments however have shown that whereas the immediate effect of high calcium concentrations on the normal heart is a systolic one the effect of the same concentrations if applied for a long time becomes a diastolic one, in other words we are able to produce with high calcium concentrations dependent on the duration of their action the same



FIG. 3. ♀ RINGER    ↑ RINGER CONTAINING 0.04%  $\text{CaCl}_2$

changes as with large and small doses of digitalis in Ringer solution. These results support the old view that calcium is involved in the action of digitalis. As to the mode of the interaction of the two substances, 25 years ago Loewi (3) expressed the opinion, based on the results of experiments performed in various lines, that digitalis changes the state of the heart in such a manner that it becomes hypersensitive to calcium and therefore reacts to the normal calcium concentration of the medium like a heart without digitalis does to high calcium concentrations. The results reported in this paper support this assumption. What other interpretation would for instance hold for the fact reported above that raising the calcium concentration after a threshold dose of digitalis in a state where this was without visible effect immediately caused a strong decrease of the systolic contractions whereas without digitalis the same calcium concentration acts only slowly and after a longer time?

Some of the results obtained make it evident that the hypersensitivity to calcium created by digitalis is not responsible for all the events to be observed as consequences of digitalis action. It may suffice to recall that the eventual stand-

still of the heart regularly happening after the application of even threshold doses of digitalis cannot be prevented by decreasing the calcium concentration of the Ringer solution. This effect must be ascribed to the same specific unknown influence of digitalis on the state of the heart which is responsible for its sensitization to calcium.

#### SUMMARY

1 Medium and large doses of digitalis applied in Ringer solution have after a short while a systolic effect, minimal effective doses after a long time a diastolic effect.

2 Raising the calcium concentration after large doses of digitalis increases after small doses decreases the systolic action.

3 The immediate action of increased calcium content of the Ringer solution on the normal heart consists in an increase of the systolic contraction—the prolonged action in a decrease.

4 Systolic or diastolic standstill of the heart as produced by digitalis cannot be produced by the application of high calcium concentration on the normal heart.

5 The mode of the interaction of digitalis and calcium is discussed.

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# THE DISTRIBUTION OF QUININE, QUINIDINE CINCHONINE AND CINCHONIDINE IN FLUIDS AND TISSUES OF DOGS<sup>1</sup>

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Of the four cinchona alkaloids currently in therapeutic use (quinine, quinidine, cinchonidine and cinchonine), only quinine has been extensively investigated with regard to the *in vivo* factors which determine the plasma concentration resulting after a given dose. The therapeutic effectiveness of a drug is usually proportional to its concentration in the plasma. Consequently these factors—the rates of absorption, excretion, metabolic alteration and the manner of *in vivo* distribution—may be as important in determining the usefulness of a drug as the host toxicity or the anti-parasitic potency.

Although these alkaloids are very similar in chemical structure, they may behave quite differently *in vivo*. It has been shown that there are marked differences in the plasma concentrations attained in man after single doses of equal amounts of the four alkaloids given orally (1). It is not yet known which of the above-mentioned factors is chiefly responsible for these differences. The investigations described here examine the factor of *in vivo* distribution particularly the partitioning of each of the four alkaloids between the plasma and tissues of the dog. This has been done recently for quinine by Kelsey, Oldham and Geiling (2).

**METHODS** *A. Experimental procedure.* Solutions of the alkaloids were infused intravenously at a constant rate for about 1½ hours. This usually served to establish a plateau in the plasma concentration for at least 30 minutes. In some experiments the animal was killed while the plasma was rising or falling to test the rate of plasma-tissue equilibration. Blood samples were taken at intervals during the course of the infusion. When the infusion was stopped the animal was killed usually by pneumothorax and samples of tissues and body fluids taken. These samples were immediately chilled and kept cold until they could be analyzed. Analysis was usually completed within a few hours after sacrificing the animal. Seven of the eleven dogs used were anesthetized with intravenously administered sodium pentobarbital. The other dogs received no anesthesia.

*B. Analytical methods.* We have adapted Brodie's colorimetric method for basic organic compounds (3) in estimating the concentration of any of the four cinchona alkaloids in tissues.

**Procedure.** Weigh out samples of tissue (usually 2 gms.) and homogenize with a small quantity of water in a glass grinder consisting of a cylindrical cup in which a close fitting

<sup>1</sup> These investigations were carried on with the aid of a grant from the Cinchona Products Institute Inc.

ground glass pestle is mechanically rotated.\* This breaks up the tissue into a soupy mash in which there are few intact cells. Transfer the mash to a flask, dilute to 20 volumes with water and precipitate with 5 volumes of 20% metaphosphoric acid. This method of precipitation, which has been utilized in the fluorometric estimation of quinine and quinidine in human plasma (4), serves to remove most of the substances contained in tissue which react with the methyl orange to give a large tissue blank. Centrifuge the precipitated tissue mash,\* pipette aliquots (usually 20 ml.) of the supernatant fluid into glass stoppered flasks and make alkaline (at least to pH 10) with NaOH solution. Add 20 ml. of ethylene dichloride to the flasks and extract the free base of the alkaloid into it by shaking the flask vigorously for five minutes. Centrifuge the contents of the flask and remove the aqueous phase by aspiration. Then shake the ethylene dichloride again with an equal volume of 10% NaOH in 20% ethyl alcohol for five minutes to remove the phenol like products of metabolic degradation of the alkaloids (3). Centrifuge and remove the upper aqueous phase by aspiration and shake the remaining ethylene dichloride with 2 ml. of a saturated solution of methyl orange in M/2 NaH<sub>2</sub>PO<sub>4</sub> for five minutes. (This dye solution must previously be extracted with ethylene dichloride until no more pigment can be removed.) Remove the excess methyl orange by centrifugation and aspiration, and transfer a measured quantity of the yellow tinted ethylene dichloride to a colorimeter tube, acidify with 0.1 part of 1% HCl in absolute alcohol, and read in the colorimeter using a 540 filter. The concentration of alkaloid is determined by reference to calibration data gained by analyzing known quantities of alkaloid in aqueous solution by the same method.

Plasma, cerebrospinal fluid, and bile were extracted into ethylene dichloride without previous precipitation. The troublesome emulsions occasionally encountered with bile were found to be prevented by adding powdered Ca(OH)<sub>2</sub> to the bile before it was shaken with ethylene dichloride. Whole blood was precipitated in the same manner as the homogenized tissues. Red cell concentrations were calculated using the plasma and whole blood concentrations as analyzed, together with the hematocrit reading.

Recovery of the alkaloids added to homogenized blank tissues and to body fluids in amounts within the range of those found in the tissues of the infused dogs was in no case less than 90% and in most determinations between 95% and 103%. The length of time the tissues were kept in the refrigerator before analysis had little effect on the blanks, the recoveries, or the concentration found in the experimental tissues.

**RESULTS** In general, the four alkaloids are distributed within the animal in the same manner (see table 1). They all occur in most tissues in higher concentration than in the plasma. The tissue/plasma ratios are relatively low in cerebrospinal fluid, red cells, brain, skeletal muscle and bile, but high in the glandular tissues and lung. Cinchonine shows somewhat lower ratios for the latter tissues than the other three alkaloids.

The tissue/plasma ratios remained fairly constant in spite of considerable variation in the plasma level. Furthermore, in the experiments where we allowed the plasma concentration to rise steeply to the lethal point (Dog No. 14), or stopped the infusion and allowed the plasma level to fall for an hour (Dog No. 13), there was little change in the ratios. These observations indicate a ready equilibrium between plasma and tissues.

When equivalent amounts of the different alkaloids were administered to dogs, either orally or intravenously, the plasma concentrations attained were approximately the same for all four alkaloids.

\* Such a grinder can be purchased from the Scientific Glass Apparatus Company, Bloomfield, New Jersey.

\* Filtration must be avoided because of the adsorption of these alkaloids on filter paper.

Sodium pentobarbital anesthesia had no consistent effect on the distribution of any of the alkaloids (table 1).

**DISCUSSION** The distribution of the four alkaloids between cells and fluids in the dog is approximately the same. It is not yet possible to say to what extent these observations can be applied to man. The fact that all four alkaloids appear in the plasma of the dog in approximately equal concentrations after equivalent doses while in man they show marked variation demonstrates a difference in the two species in one or more of the factors which affect the plasma

TABLE 1  
Tissue plasma ratios of cinchona alkaloids in the dog

	QUININE		QUINIDINE		CINCHONINE		CINCHONINE		CINCHONINE		CINCHONINE		
	Dog number												
	12 <sup>a</sup>	11	13 <sup>b</sup>	12 <sup>a</sup>	9 <sup>b</sup>	20 <sup>a</sup>	4 <sup>a</sup>	3 <sup>b</sup>	2 <sup>a</sup>	17 <sup>a</sup>	14		
Plasma conc. (mg./liter)													
	1.2	2.8	5.9	3.8	3.3	2.7	3.18	3.0	3.0	7.8	46.8		
Tissue/plasma ratios													
Cerebrospinal fluid	0.1	0.3	0.3	0.3	0.3	0.3	0.5	0.0		0.3	0.843		
Red cells	1.0	1.9	1.7	1.1	3.3	1.43	1.5	0.3		0.5	2.0		
Skin													
Brain	2.0	2.2	4.5	0.6	1.9	3.9	5.1	0.7		1.18	3.8	4.7	
Skeletal muscle	3.2	3.6	5.3	1.3	3.0	3.07	0.9	1.0		1.0	1.1	2.2	
Bile	2.3	6.3	4.4	2.0	4.8	3.0	2.4	4.0		2.6	3.2	1.0	
Intestine	5.6	3.9	9.6	7.1	6.4	6.8	8.0	3.3		5.3	5.5	3.6	
Cardiac muscle													
Spleen	29.8	76.2	22.0	19.8	22.7	16.5	27.0	11.5		6.6	6.2	4.6	
Thyroid	23.8	10.5	12.6	22.0								6.3	
Adrenals	18.2	22.1	18.6	23.2	22.0	13.7						10.0	
Kidney	11.5	22.9	20.1	20.0	18.0	13.3	19.0	7.0		13.2	14.9	10.9	
Pancreas	34.6	36.0	20.8	18.1	13.0	18.0	21.6	9.5			8.8	12.4	
Liver	19.6	22.2	14.8	39.0	17.0	17.4	34.6	14.7		13.1	13.8	15.9	
Lung	41.0	34.6	26.7	25.0	14.7	20.0	76.0	20.9		15.5	18.0	16.5	

Under nembutal anesthesia

<sup>b</sup> Without anesthesia.

concentration. In the dog the four alkaloids are handled in approximately the same manner but in man there is a marked difference in the way the four alkaloids are treated.

The accumulation of the alkaloids in the tissues probably represents a combination of the alkaloids with some constituents of the tissue cells (similar to the binding with the plasma proteins) so that they are no longer diffusible. Hatcher and Weiss (6) suggested that quinine is taken up by the capillary endothelium basing their theory on the observation that a considerable fraction of injected quinine can be recovered by perfusing an exsanguinated animal with

saline. This does not seem good evidence, because quinine could enter the perfusion fluid from the tissues. The relatively small alkaloid binding capacity of red cells, brain, and skeletal muscle, as compared with the high binding capacity of the glandular tissue, would suggest combination with some nuclear material. It is interesting to note that the degree of tissue fixation of atabrine is many times greater than that of the cinchona alkaloids, but the same difference between muscle and glandular tissues is found (7).

It must be realized that most of the alkaloid in the plasma is bound to plasma proteins, so that the diffusible fraction found in the interstitial fluid and free to enter cells may be only 30% of the total plasma concentration (5). (It may be that the cerebrospinal fluid concentrations closely approach the concentration of diffusible alkaloid in the plasma.) Furthermore, the tissues contain a certain amount of interstitial fluid, lower in alkaloid concentration. Consequently the ratio between the cellular concentration of alkaloid and the interstitial fluid concentration is actually several times greater than the tissue/plasma ratios we have reported.

In essence, we can say that any marked differences in their activity in the dog could be attributed to differences in their effects on specific cellular processes, rather than to differences in any of the factors which determine the plasma concentration attained after a given dose. Although these data are strictly applicable only to the dog, the same principles governing the plasma concentration apply to mammals generally. It is to be expected that different species will show variation in some of the factors, for example, in metabolic destruction, resulting in a different balance. An understanding of the factors determining the plasma concentration of these alkaloids is essential in approaching the problems of therapy presented by malaria and certain abnormalities of muscular function.

#### SUMMARY

1. Dogs were given infusions of the sulfates of quinine, quinidine, cinchonine and cinchonidine so as to obtain a concentration plateau in the plasma for at least 30 minutes. The body fluids and tissues were then analyzed for their alkaloid content.

2. The ratio of tissue concentration/plasma concentration was low (0.1-5.0) for red cells, cerebrospinal fluid, skeletal muscle and brain, and high (10-40) for glandular tissues and lung. Cinchonine ratios for glandular tissues and lung were somewhat lower than the other alkaloids.

3. These ratios remained approximately the same in spite of changes in the plasma concentration. Equilibration between tissues and plasma takes place rapidly.

4. With equivalent intravenous doses of the four alkaloids the plasma concentrations were approximately the same. The difference from man in this regard is pointed out.

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Since submitting this paper for publication we have discovered the omission of reference to an important paper on the concentration of quinidine in the heart and other tissues by S A Weissman in the *Am Heart Jour* 20 21 1940. Another pertinent paper by Oraham Chen and E M K Gelling on the fate of injected quinino and atabrine has recently appeared in *This Journal* 82: 121 1944

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# THE MODE OF ACTION OF NITRIC ESTERS<sup>1</sup>

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In a recent number of this Journal, Krantz, Carr, Forman and Cone (1) bring evidence "to indicate that the action of certain organic nitrates is not dependent on their hydrolysis and nitrite formation but rather upon the unhydrolysed molecule" They employed the nitric esters of glycerol, mannitol and erythritol, isomannide dinitrate and the nitric esters of some alkyl glycolic acids Still more recently Roth and Krantz (2) have shown that an effective amount of nitrite is never present in the blood after the administration of erythritol tetra-nitrate, isomannide di-nitrate, or glycerol tri-nitrate

That nitroglycerin acted through decomposition products was suggested by Onsum (3) (1865) and somewhat later by Eulenberg (4) (1865), but it was Matthew Hay (5) (1883) who placed the hypothesis on a scientific basis He showed that solutions of caustic alkalies and even of alkaline salts reduced nitroglycerin to an alkali nitrite and other products About two-thirds of the nitroglycerin was reduced to nitrite In a later paper he says "all compounds of nitric acid, whether ethereal or metallic, are without effect unless it so happen that the constitution is such that it decomposes in the body with the liberation of nitrous acid" (6) Atkinson (7) and later, Haldane, Makgill and Mavrogordato (8), mainly owing to the difference in toxic effects of nitroglycerin and sodium nitrite, threw doubts on this explanation My own investigations of the nitrates and organic nitric esters commenced in 1892 and soon I came to the conclusion that nitroglycerin and certain other nitric esters did not act, even as vasodilators, by being converted into nitrites in the body In 1897 I wrote "from my own observations I am inclined to think that nitroglycerin acts as such At least any transformation that occurs is brought about in the tissue cells themselves" (9) The main reasons were the discrepancy in the dose of sodium nitrite and of nitroglycerin to produce similar vasodilating effects, the rapidity of action of nitroglycerin which seemed to preclude any change of the nature of hydrolysis, and the different activity curve of the vasodilating action on man Hay (10) recognized the difficulty of difference of dose and suggested that the nitrous acid liberated from nitroglycerin was in a nascent state whereas sodium nitrite taken by the mouth would be converted into nitrous acid in the stomach and be decomposed by any organic matter there The suggestion, however, does not explain the greater activity of nitroglycerin when given subcutaneously or intravenously or its much greater dilating power when perfused through isolated blood vessels In an experiment on a terrapin in which the blood vessels were washed out with acidulated saline (1 in 60,000 acetic acid) dilatation was obtained when nitroglycerin (1 in 10,000) was added to the per-

<sup>1</sup> This paper is based largely upon experiments described in my M D Thesis (Univ March 1899) except the parts on oxidation and reduction and observations on the blood which have been made at various periods during the past fifty years

fusion fluid. There could be no alkaline influence in this experiment and no nitrite (starch mucilago potassium iodide, glacial acetic acid test, sensitive to 1 in 2 millions nitrite) was found in the fluid perfused. The same reagent showed absence of nitrite in many other perfusion experiments on cold-blooded animals with nitroglycerin and other nitro esters. Again, the saponification of nitroglycerin at the pH and temperature of the blood is very slow, yet in travenous injection of nitroglycerin causes an immediate fall of blood pressure due to vascular dilatation. And methyl nitrate, which is not hydrolysed to nitrite by alkalies, causes an immediate fall of blood pressure on intravenous injection. Quantitatively there is a great difference in the effect of nitroglycerin and sodium nitrite on blood vessels as shown by perfusion experiments on warm blooded animals. On perfusing a sheep's kidney under constant pressure, 1 in 5000 nitroglycerin in blood increased the venous outflow from 20 cc per minute to 63 cc per minute during the second minute on first perfusion and from 18 cc per minute to 60 cc. in the second and 70 cc in the third minute on second perfusion. Such a powerful effect is not obtained from the perfusion of nitrite solutions five times as strong. Whether the change inducing relaxation of the muscle cells is the same in the case of nitrites and nitro esters is an interesting but as yet unsolved problem. Experiments on the isolated voluntary muscle of frogs did not help in its solution but since both classes of substances affect the oxyhemoglobin of blood the action on this pigment seemed worthy of investigation in an attempt to elucidate their mode of action. Before describing the actions on blood some chemical experiments bearing on the question will be considered.

**SAPONIFICATION, OXIDATION AND REDUCTION.** Saponification. Most of the 33 nitro esters prepared by me during the last and the early years of the present century were subjected to saponification with caustic alkalies and alkaline salts. A few points only need be mentioned. Methyl nitrate saponifies normally and no reduction to nitrite occurs and only small quantities of nitrite are formed in the saponification of its higher homologues ethyl and propyl nitrates (cf. also Carlson (11)). The nitro esters of the polyhydric alcohols and those of the sugars yield nitrite readily on saponification with caustic alkali. And it is interesting that ethylene-glycol dinitrate, glycerol trinitrate and erythritol tetranitrate show diminishing stability towards the action of alkalies. The difference may be observed in the action of 1 per cent alcohollic potash on 1 per cent alcoholic solutions of glycol dinitrate and of erythritol tetranitrate respectively. At room temperature the erythritol nitric ester gave eight times the amount of nitrite given by the glycol nitric ester. Broadly the results of saponification vary with different nitro esters, with the amount and concentration of the alkali and with the temperature to which the mixture is raised. Except for small quantities of less nitrated compounds the products of hydrolysis are not of much pharmacological interest. Berl and Delpy (12) isolated from the saponification of nitroglycerin—nitrate, nitrite, alkali-cyanide, ammonia,  $\alpha$ -glycerol dinitrate, mesoxalic and oxalic acids, carbon dioxide and volatile acids and aldehydes not characterised. Some nitroglycerin was left unsaponified.

It is questionable if saponification has any place in the pharmacological action of nitric esters. It will be considered later in the case of erythritol tetranitrate. Differences in the pharmacological action of nitric esters are due more to differences in solubility and to some extent in constitution, points dealt with later. In the intracellular reactions of nitrites and organic nitrates oxidations and reductions are probably of more importance than saponification.

*Oxidation and reduction.* Inorganic nitrate is known to be reduced in small measure to nitrite by tissues (Abelous and Gerard (13)) an action attributed by Bernheim and Dixon (14) to enzymes. Stepanow (15) showed that any nitrite present in the animal body was derived from nitrate taken in the food. Pharmacologically the reduction is only of importance in showing the ability of vital tissues to effect reduction of such a stable ion as nitrate. Whether the more labile and powerful nitric esters are similarly affected by living cells has not been definitely proved, my own experiments limited to frog's muscle cells showed no such reduction. Organic unlike inorganic nitrates are readily oxidized. The oxidation products—for the most part the corresponding acids—are not of importance in the present discussion. It is however of interest to note that nitrite and organic nitrates do not react in the same way to oxidizing agents. A comparison of their effects on solutions of potassium permanganate affords an example. In acid solution nitrites decolorize potassium permanganate immediately, whereas nitric esters, whether of monohydric or of polyhydric alcohols, induce a gradual change extending over many minutes and the production of a red colour, and a permanganate solution saturated with sodium bicarbonate also reacts differently in the two cases. The reaction of organic nitrates with hydrogen peroxide is referred to later.

**ACTION OF SODIUM HYDROSULPHITE,  $\text{Na}_2\text{S}_2\text{O}_4$ , ON NITRITES AND ORGANIC NITRATES.** Moderate amounts of sodium nitrite and sodium hydrosulphite in aqueous solution react vigorously. The temperature of the mixture rises considerably in the first two minutes and the previously acid solution then begins to become alkaline. Later sodium sulphite and sulphate can be crystallized from the solution. The mother liquor contains sodium hydroxide, sodium nitrite and sodium hydrosulphite. Intermediate compounds are probably formed but no evidence of hydroxylamine or of hyponitrites was obtained. The nitrite molecule is made much more labile by the reducing agent and there is a notable increase in nitric oxide pressure even in alkaline solution. The pressure of nitric oxide in the solution is increased whether the nitrite or the hydrosulphite is in excess (Marshall and Marshall (16)).

The action of sodium hydrosulphite on nitric esters is less intense, due, at least in part, to their smaller solubility in water. These solubilities are given later. After adding 5 g sodium hydrosulphite to 1.5 g glycol dinitrate in 50 cc of water and shaking frequently no rise of temperature occurred. The hydrosulphite was converted to acid sodium sulphite in 3½ hours at room temperature and in 2 hours at 30°. No nitrite was formed. In a similar experiment in N/10 sodium hydroxide sodium sulphite was formed and the reaction was much slower. Three days after mixing sodium hydrosulphite was present al-

though undissolved glycol dinitrate lay at the bottom of the mixture. Some nitrite was also present but not more than could be explained by the saponification induced by the alkali. In N/50 caustic soda some acid sodium sulphite was formed. The reactions with methyl nitrate were weaker but otherwise similar. No nitrite was found but after standing a fortnight a trace of ammonia was present. (The test used for nitrite in these experiments was the potassium ferrocyanide acetic acid one which although relatively insensitive is much less affected than more sensitive tests by sulphites which were always present in these mixtures in considerable concentration.)

A small pressure of nitric oxide is produced by the action of sodium hydro-sulphite on organic nitrates but it is less than that obtained from nitrites. A little sodium hydrosulphite added to a saturated aqueous solution of methyl nitrate or glycol dinitrate followed by the addition of a dilute ferrous sulphate solution causes a brown coloration in a few minutes except at the surface. The colour increases somewhat in intensity and if the mixture is shaken and made uniform a colourless surface layer again develops. This colourless surface layer increases in depth until the whole mixture is colourless. Unlike nitrites organic nitrates do not form any measurable gaseous products when treated with sodium hydrosulphite and a dilute mineral acid.

**ACTION ON BLOOD** All sufficiently soluble nitric esters of polyhydric alcohols form methemoglobin if given sufficient time when added to a solution of blood. Its formation with the slightly soluble organic nitrates may also be obtained if measures are employed to increase their solubility. Nitroglycerin and some other organic nitrates often give a clearer methemoglobin spectrum than do nitrites mainly because they precipitate serum proteins an action which is probably physical since the precipitated protein contains no nitrate. With the more soluble liquid compounds hematin is eventually produced as with nitrites.

The action of sodium nitrite on blood is characterised by an induction period, a reactionary period and a stationary period and the effect is due to the hydrolysis of nitrous acid and to a pressure of nitric oxide (Marshall and Marshall (16)). The pressure of nitric oxide in solutions of sodium nitrite is small and N/100 solution is required to form any nitric-oxide hemoglobin. To transform all the pigment to nitric-oxide-hemoglobin a reducing agent is necessary (Marshall and Marshall (16)). Organic nitrates differ from nitrites in not forming NO hemoglobin unless a reducing agent is added.

**Reaction velocities** The course of the reaction on blood of nitrites and of organic nitrates is different and is shown in figure 1 which compares the effect of sodium nitrite and nitroglycerin of similar concentration on the same ox blood at neutrality (experiment made 4/5/1917). The reaction with N/300 sodium nitrite has an induction period of 10 minutes and a reactionary period of 6½ minutes that with M/200 nitroglycerin has no lag period and the curve presents more the appearance of the hydrolysis of an ester. All other experiments made with glycol dinitrate or nitroglycerin show the same type of curve. The observations were made with a HÜFNER Spectrophotometer, the eyepiece of which was adjusted to cover  $\lambda$  571-577 m $\mu$  of the spectrum. Curve C is of M/4

methyl nitrate acting on my own blood. The reaction of organic nitrates with blood rapidly falls off with increasing dilution. M/16 methyl nitrate on my own blood required two hours to complete the reaction. With nitroglycerin the red band of methemoglobin was detected in 1 hour with M/50 nitroglycerin and in 26 hours with M/800 nitroglycerin in the same specimen of blood. It is common experience that the blood of different animals and blood under different conditions react somewhat differently to nitrates in time and degree. My impression is that the same is true of organic nitrates although to a less extent.

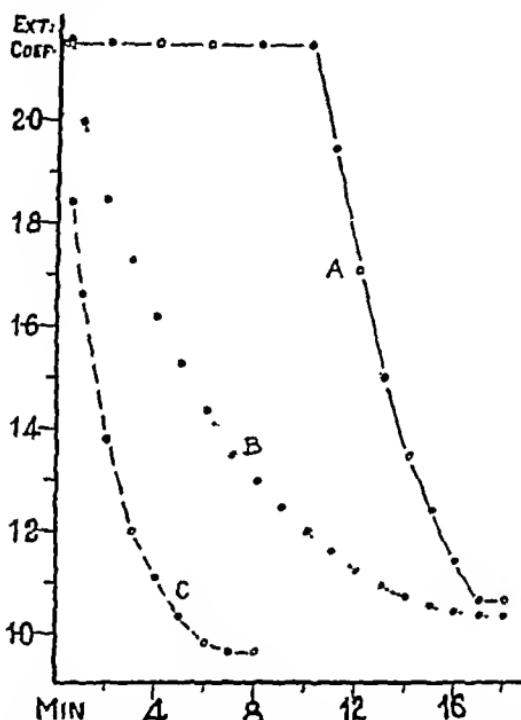


FIG 1 Graphs of the velocity reactions of A N/800 sodium nitrite on 2 per cent ox blood two days old B M/200 nitroglycerin on the same blood C M/4 methyl nitrate on my own blood, probably about 15 per cent strength. Ordinates = extinction coefficients. Abscissae = time in minutes. The experiment with nitroglycerin is the best obtained. Often the reaction is slower.

*Effect of pH.* Variations in pH have much less effect on the action of nitric esters than of nitrates. With the latter the effect of pH is profound, especially on the changes occurring during the induction period. The normalities of sodium nitrite from pH 5 to pH 9 necessary to induce a reaction commencing at a definite interval appear to form a geometrical series (Marshall and Marshall (16)). An experiment with methyl nitrate will suffice to show the different influence of pH on organic nitrates. The methyl nitrate was added to a solution of blood buffered to pH 5.2, 6.0, 7.2, 7.6, 9.2 and kept at room temperature. In pH 5.2 the oxyhemoglobin was converted to methemoglobin within an hour, in pH 6.0 it was only partially changed to methemoglobin in an hour but was

wholly changed in two hours in pH 7.2 only a small change occurred and at pH 7.6 a just detectable change occurred in two hours, in pH 9.2 no change was noted at this time but a nearly complete transformation appeared to have occurred in nineteen hours. On adding a little sodium hydrosulphite to each mixture they all changed colour to rose-red within a few minutes—2 to 3 minutes for the more acid, 5 minutes for the most alkaline—at the end of which time the colour of the several mixtures was indistinguishable. The last change is similar to that occurring with nitrites. Thus it would appear that the differences in action between nitrites and organic nitrates is connected with the method of forming methemoglobin which in the case of nitrites seems to be associated with the hydrolysis of nitrogen tetroxide whereas in the case of organic nitrates it is due to some other effect not yet determined.

The nitrites and organic nitrates show differences in some reactions involving hydrogen peroxide on blood and a similarity in a reaction with ammonium sulphide which is interesting in that it is not given by other substances producing methemoglobin. The latter will be described first.

*The ammonium sulphide reaction.* Gamgee (17) who first described the action of nitrite on blood, states that when ammonium sulphide is added to nitrited blood the first change is to oxyhemoglobin then to reduced hemoglobin which however can be reoxidized by shaking with air. The reduction and oxidation can be observed several times in the first few minutes. Later and thus result Gamgee does not describe, NO-hemoglobin is formed (8). Methemoglobin prepared by the action of organic nitrates reacts in the same way. The only difference observed was a slower change to reduced hemoglobin. Oxyhemoglobin is not formed when ammonium sulphide is added to a methemoglobin solution produced by potassium ferrocyanide, quinone acids or other reagents. Its formation in the case of nitrited blood has not been explained but as it only results from the use of ammoniacal reducing agents (ammonium sulphide and Stokes' reagent) it may be connected in some way with the ammonia which has an inhibiting effect on the action of nitrite on blood (Marshall and Marshall (18)).

*A hydrogen peroxide reaction.* By the addition of the appropriate amount of hydrogen peroxide to a methemoglobin solution produced by the action of sodium nitrite, a spectrum showing a single band about the D line forms, which on the addition of sodium hydrosulphite is quickly changed to a spectrum with a single band about the position of the methemoglobin band in the red. In a short time two weak bands, probably of NO-hemoglobin, appear in the yellow green. The bands are unaffected by further addition of sodium hydrosulphite (Marshall and Marshall (18)). Methemoglobin solutions formed by adding organic nitrates to blood do not pass through these changes. The addition of peroxide in effective quantities converts the methemoglobin to oxyhemoglobin as it does the methemoglobin produced by quinone and acids. Ferrocyanide methemoglobin forms an unstable compound with hydrogen peroxide (Keilin and Hartree (18)) in the absence of catalase activity. It is interesting to note that nitrites inhibit catalase action organic nitrates do not, at least not to any thing like the same extent.

**SOLUBILITY AND ABSORPTION** The solubilities in water of the type substances to be considered in this section are, for 100 cc. water at 18°C. Methyl Nitrate, 3.92 g., Ethylene-glycol Dinitrate, 0.53 g., Glycerol Trinitrate, 0.12 g., Erythritol Tetranitrate, 0.005 g., Mannitol Hexanitrate, less than 0.001 g. Owing to the very slight solubilities of the two last, an indirect method had to be used to determine them. A filtered saturated solution was saponified by caustic potash and the solubility calculated from the nitrite formed using the nitrite number in alcoholic solution previously determined.

If methyl nitrate be excluded for the present and attention be confined to the nitric esters of polyhydric alcohols, the intensity of the effect for oral administration is in the order of the solubility of the substances. The more soluble glycol dinitrate and glycerol trinitrate are sufficiently volatile to produce a pharmacological effect and both are lipid-soluble and are quickly absorbed from the stomach. Still a large number of experiments on men showed that glycol dinitrate is somewhat more rapid and transient in action than is glycerol trinitrate. Both are more powerful but much more transient in action than erythritol tetranitrate which in turn is more powerful than mannitol hexanitrate.<sup>2</sup>

The parallelism of activity and solubility is equally well shown in the toxic effects on animals. The lethal dose by oral administration varies somewhat with the presentation. A dose of 0.5 g. per kg. body-weight glycol dinitrate when merely shaken up with water and given to rabbits by the stomach tube is not fatal but when made into an emulsion with a little mucilage it quickly produces serious symptoms and death. If the rapidity of absorption is increased by the addition of a small quantity of alcohol death also follows. After a dose of 1 g. per kg. body-weight death results however the drug is administered. The less soluble nitroglycerin is less toxic when given by the stomach. The following experiment illustrates the point. Two rabbits were taken, to one was given glycol dinitrate (0.5 g. per kg. body-weight), to the other glycerol trinitrate (0.75 g. per kg. body-weight). Both recovered, the following morning they were apparently normal. Two days later the same rabbits were used and the administration reversed. To the nitroglycerin rabbit glycol dinitrate (0.75 g. per kg. body-weight) was given, to the other glycerol trinitrate (0.5 g. per kg. body-weight). The first rabbit died having shown the usual symptoms common to these substances—dilatation of the blood-vessels, more frequent pulse, some dyspnoea, methemoglobinemia, muscular weakness and fall of temperature—although two days previously a similar dose of nitroglycerin had produced only slight effects. The second rabbit seemed scarcely affected by the smaller dose of nitroglycerin—the rectal temperature fell from 38.4°C to 37.4°C. The same dose of glycol dinitrate administered to the same animal two days

<sup>2</sup> Some investigators have found mannitol hexanitrate as powerful as erythritol tetranitrate. This finding must have been due to the use of impure mannitol hexanitrate. I have prepared many batches of this compound and have never obtained it pure by ordinary nitration, even when using the strongest nitric and sulphuric acids available. The product always contained the much more soluble and more active pentanitrate. For the pharmacological action of mannitol pentanitrate see Brit. Med. Journ. Oct. 18, 1902.

previously had caused the temperature to fall from 38.5°C to 32.7°C and had produced marked dilatation of the blood vessels of the ear methemoglobin in the blood dyspnoea and decided muscular weakness.

The solid organic nitrates in single doses given mixed with water by the mouth produced no obvious toxic symptoms in rabbits. Erythritol tetranitrate in doses of 2 g per kg body weight and mannitol hexanitrate in doses of 3 g per kg body weight induced no symptoms whatever but continued administration of much smaller doses twice daily of erythritol tetranitrate will produce a lethal effect after some days. That the comparative inactivity of the solid organic nitrates is due to their insolubility is shown by the fact that fish—newly hatched trout or salmon are best for such experiments—allowed to swim in solutions of nitroglycerin and of erythritol tetranitrate of equal strength are somewhat more readily killed by the latter solution although fish swimming in nitroglycerin solution show the first symptoms.

That the differences described are largely due to differences in absorption resulting from differences in solubility may be seen in the effect on blood pressure of similar concentrations intravenously injected. The effect of nitroglycerin on blood pressure as illustrated in a tracing chosen from my collection by Professor Cushny for his article in Heftner's Handbuch der exper. Pharmak. I 833 was preceded by the injection of the same amount of erythritol tetranitrate under identical conditions six minutes earlier. The two tracings are so similar that they may be superposed.

Another class of experiment allowing of comparison is that of the perfusion of isolated blood vessels when the same concentration has been used. Unfortunately second perfusions of the same organ are usually less reactive than first perfusions so that comparison must be limited to the latter and again exact comparison is precluded by the fact that the tone of the vessels to be perfused is not always the same. For the perfusion of warm blooded organs under constant pressure the kidney proved best for my experiments. Table 1 gives the outflow in cc., constant for four successive minutes and therefore termed normal of the animal's own blood perfused through the kidney of a sheep under a pressure of 70 mm Hg and the outflow during the third minute of perfusion of 1 in 10 000 of the compounds named. Experiments with some nitro-sugars to be mentioned later are included for comparison. Broadly these experiments show that when fully nitrated compounds of polyhydric alcohols and sugars come into direct contact with the tissue they predominantly influence, their effect is similar even quantitatively. The higher molecular compounds appear to penetrate into cells somewhat less readily than the lower a point referred to in the next section.

This paper is chiefly concerned with combatting the view that organic nitrates act pharmacologically through the mediation of nitrites. It may be recorded that the slightly soluble solid organic nitrates were investigated on the assumption that they would yield by saponification in the intestine a continued small amount of nitrite. Experiments failed to support the view. After large doses of organic nitrates by the mouth to rabbits a small amount of nitrite

along with much unchanged nitric ester can be extracted from the urine after a few hours and for a day or more, but in my experiments with erythritol tetranitrate the amount of nitrite in the urine has been less than after the administration of nitroglycerin. When small doses of erythritol tetranitrate are given repeatedly, larger quantities of nitrite are found in the urine. Thus in an experiment in which 0.1 g. erythritol tetranitrate was given night and morning to a guinea pig, more nitrite than unchanged nitrate was obtained. This result was the only positive evidence in support of the theory. Analyses of the intestinal contents after any doses failed to show distinct amounts of nitrite. Even the relatively large dose, compared with that of nitroglycerin, of erythritol tetranitrate necessary to produce a pharmacological effect on man, would if wholly transformed to nitrite, be quite inadequate to produce the effect obtained. Roth and Krantz's experiments (2) showing that the nitrite level in the blood was not increased at the time of the fall of blood pressure produced by organic nitrates is supporting proof of their independent action. Hay's theory of the

TABLE 1  
*Perfusion through kidney of sheep under pressure of 40 mm. Hg*

COMPOUND	NORMAL OUTFLOW PER MINUTE IN CC.	OUTFLOW DURING THIRD MINUTE OF PERFUSION 1 IN 10 <sup>4</sup> OF SUBSTANCE
Glycol dinitrate	9	23
Glycerol trinitrate	8½	19
Erythritol tetranitrate	13	85
Mannitol hexanitrate	6	11
Glucose pentanitrate	14	22
Quercite pentanitrate	22	37
Saccharose octonitrate	12	24

action of organic nitrates would thus seem to have little pharmacological support.

**NERVOUS ACTIONS** Effects which are of interest in a comparison of the action of nitrite and organic nitrates are exhibited on the central nervous system. Albers (19) (1864) records that nitroglycerin produces in frogs muscular stiffness and convulsions and his observations have been corroborated by many subsequent researchers. Brunton and Tait (20) attribute the effects to an action on the optic lobes. Atkinson (21) regards the tetanus as of spinal and the clonic convulsions as of medullary origin. I came to the conclusion that the cause of the convulsions was both basal and spinal. But the seat of action is not material to the present discussion. It is an action not induced by nitrites which cause, not stiffness and convulsions, but paralysis, in toxic doses. The dose necessary is approximately about ten times that of nitroglycerin required to produce convulsions.

After the injection of 1 mgm. nitroglycerin into a lymph sac of a frog about

20 g weight, muscular stiffness is demonstrable in about 5 minutes and convulsions, usually of tonic type, soon follow and recur at intervals during the succeeding 10 minutes. Stiffness of movement may be observed for 2 hours or more, after which time recovery quickly occurs. Convulsions are not produced in similar frogs by the injection of 4 mgm glycol dinitrate or by 20 mgm methyl nitrate. Slight muscular stiffness may be observed but flaccidity, especially after methyl nitrate, is a more prominent symptom.

In the case of the less soluble organic nitrates no symptoms were produced in frogs by any dose that could be injected into the lymph sacs. As it was desirable to know if this action was induced by the higher nitrated members of the series experiments were made on fish, mainly newly hatched salmon and trout and on tadpoles. Nitroglycerin, 1 in 100 000 solution will induce convulsive movements in about 10 minutes, 1 in 10,000 in 2 minutes and death in a few hours. Erythritol tetranitrate 1 in 100 000 solution, did not cause convulsions but 1 in 20 000 solution induced convulsive twitchings of the tail in 10 minutes and more prolonged contractions later. In comparable concentrations convulsive twitchings or contracture appeared decidedly later in erythritol tetranitrate solutions than in nitroglycerin solutions although the erythritol nitrate appeared to be the more lethal. Saturated solutions of mannitol hexanitrate had no obvious effect. It would seem that erythritol tetranitrate is less readily absorbed but is effective in inducing convulsive movements after absorption and that still less soluble higher nitrated compounds are not absorbed in effective amounts to produce this action.

The question whether these nervous effects are due to the alkyl radical or to the nitrate moiety suggested itself. Glycerin does not produce them but it is not a good comparative substance for nitroglycerin. Glycerol-glucoside as more likely to give information of the action of the glyceryl group was prepared but on testing on frogs it did not produce the characteristic nervous effects. Nor did the corresponding hydrochloric ester tri-chlorhydrin.

**CHEMICAL CONSTITUTION AND PHARMACOLOGICAL ACTION.** The nitro esters investigated by me excluding insoluble compounds and the nitro esters of some organic acids, fall pharmacologically into two main groups (i) Those saponified normally by alkali into inorganic nitrate and the corresponding alcohol, like methyl nitrate and the immediate higher homologues and (ii) those forming nitrite and other products on saponification with alkalies, like the nitric esters of the polyhydric alcohols and the sugars. The members of the first group are less active as vasodilators than the members of the second group. The toxic effects of the two groups also show differences. The chief symptoms of poisoning by glycol dinitrate and nitroglycerin have been mentioned. Methyl nitrate given to rabbits produces the symptoms of organic nitrate poisoning—quickening of the pulse, dyspnoea, methaemoglobinæmia—and symptoms of alcohol poisoning—unsteadiness, chewing and running movements, unconsciousness. Small toxic doses (1 g per kg body weight) caused some dilatation of the blood vessels and increased frequency of the pulse and slight lowering of the temperature (0.8°C) but no dyspnoea. Chewing movements occurred but

consciousness was not lost and apparent complete recovery followed within an hour of the administration. The lethal dose for rabbits given by the stomach tube is 5 g per kg body-weight, and is approximately five times that of glycol dinitrate.

In its actions on separate tissues methyl nitrate is also less powerful than the soluble nitric esters of polyhydric alcohols. This point in the case of blood is shown in figure 1. On blood-pressure nearly fifteen times the quantity of nitroglycerin intravenously is required to produce a similar effect, and nearly fifteen times the concentration of nitroglycerin is necessary to produce a comparable vasodilating effect when perfused through the blood vessels of the sheep's kidney. In man the dose necessary, when given by the mouth, to produce a comparable vasodilator effect is one hundred times that of nitroglycerin, but its effect is more prolonged.

The homologues of methyl nitrate act similarly. There is an increase in toxicity and to a less extent in vasodilating power as the series is ascended, at least as far as the amyl nitrates. The difference in pharmacological action of methyl and ethyl nitrates is small. It is greater between these and the propyl nitrates. Propyl and iso-propyl nitrates have practically the same pharmacological action. Secondary butyl nitrate is somewhat more active than normal butyl nitrate probably owing to its lesser stability. Cash and Dunstan (22) found the same for the corresponding nitrite compounds. I was unable to prepare a tertiary nitrate for pharmacological investigation. Similar differences in activity are found in the alcohols and other comparable compounds and are attributable more to the alkyl radical and to changes in physical characters than to any influence of the nitrate group.

With the nitric esters of the monohydric alcohols penta-erythritol tetranitrate may be associated. This compound has four nitrated primary alcohol groups attached to a central carbon atom. It is a much more stable substance than erythritol tetranitrate and it has a much weaker pharmacological action.

The vasodilating action of the nitric esters of other polyhydric alcohols is connected solely with the nitrate groups. Modifications in the alkyl moiety are in general of small importance. Propylene-glycol dinitrate has the same action as ethylene-glycol dinitrate and, apart from its smaller solubility in water, so has trimethyleneglycol dinitrate. The arrangement of the nitrate groups in the nitric esters of hexahydric alcohols or sugars appears to be without influence. Mannitol hexanitrate, dulcitol hexanitrate and sorbitol hexanitrate have the same action, and arabinose tetranitrate, rhamnose tetranitrate, glucose pentanitrate, fructose pentanitrate, quercite pentanitrate and saccharose octonitrate show no differences which cannot be attributed to differences in stability and solubility. And, apart from changes in solubility, the introduction of alkyl or halogen groupings into such compounds does not appear to influence the vasodilating action. Methyl-glycerol dinitrate has practically the same vasodilating action as glycerol dinitrate, and dichlorhydrin nitrate as glycerol mononitrate.

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trations but, since the hydroxyl also confers greater solubility in water it may happen that the less nitrated compound is more active pharmacologically. Glycerol dinitrate is much less active as a vasodilator than is glycerol trinitrate and glycerol mononitrate is much less active than glycerol dinitrate. Mannitol pentanitrate on the other hand is more powerful than mannitol hexanitrate because it is at least forty times more soluble in water than mannitol hexanitrate. And dulcitol pentanitrate is more powerful than dulcitol hexanitrate for the same reason. If however, they are compared in the same concentration the pentanitrates are less powerful than the hexanitrates. In the same concentration arabitol pentanitrate is more powerful than the pentanitrates of mannitol or dulcitol.

In a series of interesting papers Krantz and co-workers (1 2 23) have described the pharmacological action of the nitric esters of the anhydrides of erythritol and mannitol, and, although much more soluble in water than the nitric esters of erythritol and mannitol have found them to possess the same prolonged action. Krantz, Carr, Forman and Ellis (24) conclude that 'the presence of the ether linkage in the molecule of the nitrates of the sugar alcohols reduces somewhat the potency of the compound as a depressor drug but prolongs the duration of response'. Isomannide dinitrate was most extensively investigated. Isomannide is a comparatively stable substance and Krantz, Carr, Forman and Ellis (25) found that isomannide dinitrate unlike the nitrates of glycerol, erythritol and mannitol, is not hydrolysed by caustic alkali. In this respect it is like the nitrates of monohydric alcohols and the description given of its vaso-dilating action seems to me to agree more closely with that of the nitrates of monohydric alcohols than with that of the nitrates of polyhydric alcohols.

Krantz, Carr, Forman and Cone (7) in explaining the action of organic nitrates invoke the Partition Law to an extent greater than I can accept. The Principle of Partition Coefficients in pharmacology is concerned mainly with absorption (through the skin or mucous membranes, or from the blood or other extra-cellular fluid into cells) and not with assimilation and it is certain that in producing their predominant action nitric esters undergo change within cells. The lipid content of unstripped muscle is not very different from that of blood plasma and consequently it is not obvious why lipid solubility should play so important a part in the action of nitric esters. A difference of partition-coefficients may explain the difference in vaso-dilator power of the nitric esters of some organic acids and their alkyl derivatives noted by Krantz, Carr, Forman and Cone (1) and also recorded by myself (26) but the vaso-dilating action of other equally comparable series such as the nitrates of homologous monohydric alcohols, is not proportional to their partition-coefficients. Moreover the position of glyceryl trinitrate, given by Krantz, Carr, Forman and Ellis (27) in table I does not seem to support their contention. Glyceryl trinitrate pharmacologically does not occupy a position between erythritol tetranitrate and mannitol hexanitrate and since it is 25 times more soluble in water than erythritol tetranitrate it is difficult to understand the partition figures given.

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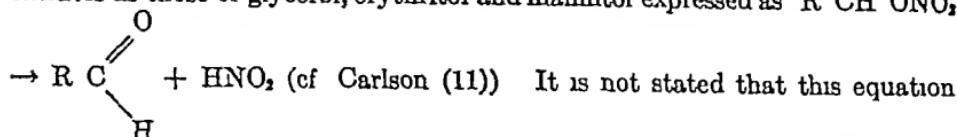
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of an intramolecular oxidation occurring after hydrolysis for such organic nitrates as those of glycerol, erythritol and mannitol expressed as R CH ONO,



represents a possible intracellular change but it is worthy of consideration from this point of view. Aldehydes do not produce vaso-dilating effects and therefore the equation if accepted requires nitric esters to act through the mediation of nitrous acid. This hypothesis involves us again in the nitrite theory but now in the cells themselves. It is a theory I once held, partly because the degree of activity of the nitric esters as vaso-dilators is roughly, although not exactly, parallel with their hydrolysis to nitrite by alkalies. But later work with blood as described in a previous section seemed to show that the dissolution of nitrite and organic nitrate is not the same, and there remains the bogie that if the whole of a therapeutic dose of nitroglycerin were converted to nitrous acid it would not explain the effect produced. The kinetics of nitrate have not proved so helpful as the kinetics of nitrite in unravelling their respective pharmacological actions. Bruhl (28), from physical experiments, and Carlson (11), from chemical ones, suggested that organic nitrates possess peroxide properties. Carlson found more oxygen was given off from a mixture of nitroglycerin, ethyl alcohol and hydrogen peroxide alkaliised with caustic soda, than from alcohol, hydrogen peroxide and alkali alone—138 cc compared with 21 cc in the control in 20 hours. My experiments with methyl nitrate and ethylene-glycol dinitrate have not confirmed his experiments with nitroglycerin. These organic nitrates did not increase materially the gas given off when alcoholic solutions were added to alkaliised hydrogen peroxide. Moreover the velocity of the reaction, which is very slow, is not distinctly affected by the presence of organic nitrates, nor have the organic nitrates in moderate quantities any decided effect on the liberation of oxygen from hydrogen peroxide by blood. It is difficult to suppress the idea that the initial pharmacological action of organic nitrates, at least on blood, is associated with a very mobile oxygen atom, but I have not been able to obtain any supporting evidence for the view.

#### SUMMARY

Saponification of organic nitrates is briefly considered and the reaction of nitrites and organic nitrates to oxidizing and reducing agents is described.

The action of nitrite and organic nitrates on blood, on certain reactions in blood, and on the nervous system are compared.

The influence of solubility on the absorption and pharmacological action of organic nitrates and of modifications in their constitution are discussed.

It is concluded that organic nitrates do not act through the mediation of nitrites.

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# SYNTHETIC BENZHYDRYL ALKAMINE ETHERS EFFECTIVE IN PREVENTING FATAL EXPERIMENTAL ASTHMA IN GUINEA PIGS EXPOSED TO ATOMIZED HISTAMINE

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Recent publications present a substantial amount of evidence favoring the hypothesis that most of the symptoms of anaphylactic shock are due to histamine liberated by the tissues during the antigen-antibody reaction (1-5). The development of chemical or biological substances capable of diminishing or abolishing some of the effects of histamine would thus be expected to alleviate anaphylactic shock and possibly various allergic manifestations in which histamine may be a causative factor.

Hill and Martin (6) published an extensive critical review in 1932 which served to emphasize the fact that although numerous substances had been tested there was none which could be considered an ideal agent for alleviating anaphylactic shock in experimental animals. During the past decade more efficacious means of antagonizing histamine have been sought (7).

The enzyme, histaminase, slowly destroys histamine *in vitro*. Claims made to the effect that parenteral injection of histaminase alleviates histamine shock and anaphylactic shock (8, 9) have not received support from data obtained by subsequent investigations (10-14).

Fell and co-workers (15, 16) demonstrated that histamine functions as a hapten when combined with despeciated normal horse serum globulin for antibodies partially specific for histamine were produced in animals, and immunized guinea pigs exhibited a definite degree of resistance to anaphylaxis. Such antibodies have also been demonstrated in human beings (17, 18).

French investigators (19-21) have selected a number of chemical compounds which possess remarkable anti-histamine potency. The ability of 2-isopropyl-5-methylphenoxyethylamine (929F) and N-phenyl-N-ethyl-N'-diethylethyl-enediamine (1571F) to alleviate anaphylactic shock and histamine-induced bronchoconstriction and to prevent histamine from contracting intestinal muscle has been confirmed (22-24). The drug, Antergan, N-dimethylaminoethyl-N-benzylaniline, with anti-histamine properties similar to those possessed by 929F and 1571F has recently been reported to be therapeutically effective in some cases of asthma, hay fever, serum sickness, urticaria, migraine and other clinical conditions (25-28).

The anti-histamine activity of an extensive series of synthetic antispasmodics was studied by testing their ability to relieve bronchoconstriction and thereby

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reduce the percentage mortality in guinea pigs treated with histamine. The majority of the compounds studied were benzhydryl alkamine ethers which can be represented by the general formula,



C—O—R where R represents a carbon chain bearing a primary, sec-

ondary or in most cases a tertiary nitrogen atom. For example, R may be  $\beta$ -dimethylaminoethyl or  $\gamma$  morpholinopropyl.

These compounds were synthesized by Dr. George Rieveschl Jr.<sup>1</sup> and Dr. W. Frederick Huber<sup>2</sup> in the laboratories of the Department of Chemical Engineering University of Cincinnati and Parke Davis & Co. Detroit. The hydrochlorides of the benzhydryl ethers are soluble in water and stable under ordinary conditions of temperature and atmosphere.

**METHODS** Inhalation of atomized histamine induces a bronchial asthma in guinea pigs which can be alleviated by anti histamine or antispasmodic drugs (13, 26, 29-31). We have produced fatal bronchoconstriction consistently in guinea pigs with rapidity and ease by subjecting them to histamine atomized under standardized conditions. Testing of compounds for anti-histamine activity consisted of comparing the incidence of mortality in guinea pigs treated with a given compound before subjecting them to atomized histamine with that obtained in untreated animals approximately 90 per cent of which succumbed within 15 minutes.

The apparatus (fig. 1) consisted of a plain cylindrical glass jar (Corning Pyrex #850) of 4.5 gallon capacity which was inverted over a carrier of galvanized wire mesh divided into four compartments each accommodating a single guinea pig. The carrier was constructed with a cylindrical opening in the center so as not to obstruct the mist which was emitted vertically from a glass atomizer fixed in the center of the inverted desiccator cover. One of the two glass tubes passing through the bottom of the chamber served as an open vent the other connected to a vacuum line, made it possible to draw air through the chamber in order to remove any histamine which might be present at the end of an experiment.

The glass atomizer constructed for use was similar to the metal atomizer described by Tattersfield (32). It was connected with rubber tubing to a mercury manometer and thence to the compressed air line the tubing being provided with an open T. The capillary tubing centrally placed in the atomizer measured 1 mm in diameter. Continuous air flow was so adjusted that closure of the T resulted in a pressure of 150 mm Hg and vaporization as a fine spray, of 0.5 cc. of aqueous histamine diphosphate within 15 to 25 seconds. Under these conditions a concentration of histamine of 1.80 consistently produced 80 to 100 per cent mortality when administered to a total of 16 to 20 untreated guinea pigs on any given day. Replacement of the atomizer necessitated re-determination of the concentration of histamine which would produce the same incidence of mortality.

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Compounds to be studied were injected intraperitoneally 15 minutes before subjecting the guinea pigs to atomized histamine solution. Animals of both sexes weighing 250 to 450 grams were used. Records were kept of onset, duration, and degree of symptoms as well as incidence of mortality. Ten minutes after the histamine was atomized a current of air was drawn through the chamber for two minutes before removing the animals.

The acute toxicity (table 1) of each compound was determined by intraperitoneal administration of a different dose to each of five groups of 10 male and female

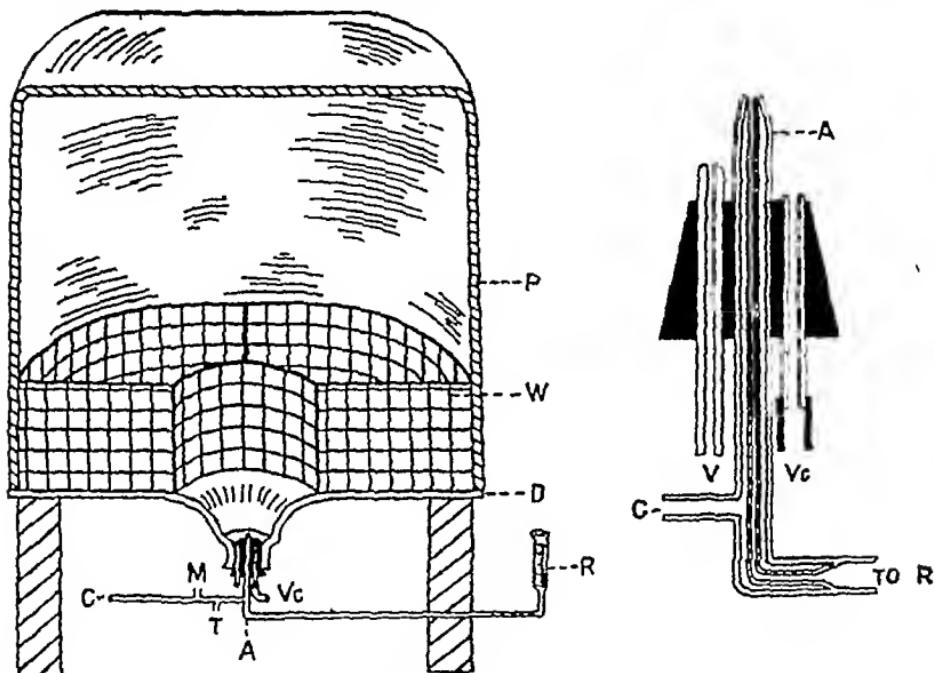


FIG 1. DIAGRAM OF APPARATUS USED TO ATOMIZE HISTAMINE AND PRODUCE FATAL EXPERIMENTAL ASTHMA IN GUINEA PIGS

P, plain glass jar (12 x 12 in.) inverted over wire carrier (W)

D, inverted desiccator cover serving as floor of chamber

A, glass atomizer (details shown at right) and graduated reservoir (R) connected with rubber tubing to mercury manometer at (M) and to compressed air line at (C)

T, open T

V, one glass tube through stopper served as a vent, the other (Vc) was connected to the vacuum line

albino rats weighing 100 to 125 grams. The deduced mortality was determined by the method described by Dragstedt and Lang (33) and later used by Behrens (34). These data were used in construction of a dosage-mortality curve from which the LD-50 was calculated.

**RESULTS** The criterion for determining whether a given drug was efficacious in alleviating bronchoconstriction was a significant reduction in percentage mortality from that which obtained in uninjected control animals subjected to atomized histamine under identical conditions on the same day.

The degree of potency possessed by a drug has been expressed by an activity

index Aminophylline at a dose of 50 mgm./kgm. reduced the mortality of histamine-poisoned guinea pigs 50 per cent and has been given an activity index of unity. The activity index of each compound tested is the ratio of the 50 mgm./kgm. dose of aminophylline to the minimum mgm./kgm. dose of a given drug which reduced mortality a significant degree. Thus papaverine at a dose of 25 mgm./kgm. reduced mortality 30 per cent and therefore has an activity index of 50/25 or 2.0.

Our data indicate that a comparatively large dose of aminophylline was required to reduce the mortality of guinea pigs subjected to atomized histamine. Young and Gilbert (35) reported that doses of 30 to 60 mgm./kgm. injected intravenously diminished the symptoms and mortality of guinea pigs injected with histamine or subjected to anaphylactic shock. Emmelin *et al.* (36) found that high concentrations of xanthine derivatives, designated as anti-histamine and anti-anaphylactic were necessary to definitely antagonize the contracting effect of histamine or antigen on intestinal muscle *in vitro*.

In sharp contrast to aminophylline and papaverine the Fournau histamine antagonists, 2-isopropyl-5-methylphenoxydiethylamino (929F) and N-phenyl N-ethyl N'-diethyl-ethylenediamine (1571F), are effective in small doses. These compounds were studied pharmacologically in some detail by Staub (21) and later by others (22-24) and there is agreement that the compounds are markedly active in counteracting the effects of histamine on smooth muscle as demonstrated by *in vitro* and *in vivo* experiments. Our data obtained with the atomized histamine technique reveal that 1571F is considerably more potent than 929F. This is in general agreement with the observations of Staub (21) who found that 929F protected guinea pigs to some degree against three lethal doses of histamine injected intravenously, whereas 1571F was effective against four lethal doses and papaverine against only one lethal dose. With the possible exception of Antergan (25, 26) 929F and 1571F are the most potent histamine antagonists heretofore described and in these experiments relatively small doses markedly reduced mortality. The maximal doses of the Fournau compounds employed were tolerated well and although the atomized histamine usually induced dyspnea very few of the animals developed asphyxial convulsions.

The benzhydryl ethers are listed in table 1 in descending order of anti-histamine potency as indicated by their effectiveness in reducing the percentage mortality in guinea pigs subjected to atomized histamine. Inspection of the accumulated data relating to the Fournau compounds and to a number of benzhydryl ethers reveals that a decrease in dosage with a given compound almost without exception resulted in a decrease of effectiveness.

The three most potent of these new synthetic compounds are  $\beta$ -dimethylaminoethyl  $\beta$ -piperidinoethyl and  $\beta$ -morpholinoethyl benzhydryl ether. The first two compounds are at least twice as potent as  $\beta$ -morpholinoethyl benzhydryl ether which has activity comparable to that of 1571F, the most active Fournau histamine antagonist included in this study. With each of these four compounds the minimum dose which reduced mortality a significant degree represents only 1/30 to 1/60 of the intraperitoneal LD-50 in rats or guinea pigs. Compound No. 2 is more toxic in guinea pigs than in rats. The highest doses employed with

TABLE 1

*Efficacy of Alkaline Benzhydryl Ethers and Amines in Preventing Fatal Histamine Induced Bronchoconstriction in Guinea Pigs*

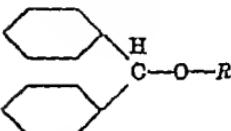
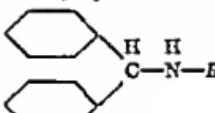
CHEMICAL COMPOUND	TREATED		UNTREATED CONTROLS		DECREASE IN PER CENTAGE MORTALITY	Pf	ACTIVITY INDEX	LD-50 (MGM./KG. I.P.)			
	Dose I.P. (mgm./kgm.)	Mortality		Mortality							
		Ratio	Per cent	Ratio	Per cent						
Reference Compounds											
Theophylline-ethyl-enediamine (Amino-phylline)	50	12/20	60	19/20	95	35	<0.01	1 252			
Papaverine	25	14/20	70	19/20	95	25	0.03	2			
N-Phenyl N-ethyl-N'-diethylethylene diamine (1571F)	25	2/20	10	20/20	100	90					
	12.5	5/20	25	19/20	95	70					
	6	8/20	40	15/16	94	54					
	3	12/20	60	15/16	94	34	0.01	16 142			
2-Isopropyl-5-methyl-phenoxyethylideneethylamine (929F)	25	1/20	5	20/20	100	95					
	12.5	2/20	10	19/20	95	85					
	6	15/20	75	16/16	100	25	0.01	8			
Benzhydryl Ethers											
											
1 R = $\beta$ Dimethylaminoethyl	12.5	0/20	0	19/20	95	95					
	6	2/28	7	19/20	95	88					
	3	9/20	45	10/20	95	50					
	1.5	7/20	35	18/20	90	55	<0.01	33 82 (75)‡			
	7.5	17/20	85	23/24	96	11	0.2				
2 $\beta$ Piperidinoethyl	12.5	2/20	10	18/20	90	80					
	3	4/20	20	13/16	81	61					
	1.5	15/20	80	15/16	100	20	0.03	33 80 (42)			
8 $\beta$ Morphinolinoethyl	12.5	5/20	25	19/20	95	70					
	6	7/20	35	10/20	95	60					
	3	30/40	75	35/36	97	22	<0.01	16 185 (180)			
4. $\beta$ Methylaminoethyl	12.5	2/16	13	35/40	88	75					
	6	2/8	25	19/24	79	54	<0.01	8 85			
	3	10/20	65	18/20	90						
5 $\beta$ Isopropylaminoethyl	12.5	5/20	25	18/20	90	65					
	6	13/20	65	18/20	90	25	0.05	8 72			
6 Diethylaminoethyl	12.5	9/20	45	18/20	90	45					
	6	14/20	70	20/20	100	80	<0.01	8 85			
7 Diethylaminoethyl	12.5	7/20	35	20/20	100	65	<0.01	4 55			
	6	16/20	80	19/20	95	15	0.15				

TABLE 1—Continued

CHEMICAL COMPOUNDS	TREATED		UNTREATED		DECREASE IN PER- CENTAGE MORTALITY	P†	ACTIV- ITY INDEX	LD-50 (Dose/ kgm.‡)			
	Dose, I.P. (mgm./ kgm.)	Mortality		Mortality							
		Ratio	Per cent	Ratio	Per cent						
8. $\gamma$ -Morpholino- propyl	25 1. 6	15/20 12/20	47 60	19/20 19/20	90 95	43 35	<0.01	4			
9 $\beta$ -Aminoethyl	25	14/20	0	16/16	100	30	<0.01	2			
10 $\beta$ -( $\beta$ -Morpholino- ethylamino)ethyl	25	8/16	50	10/24	79	29	0.04	2			
11 4,4-Dichloroben- zhydryl $\beta$ -morpho- linoethyl ether	25	12/20	60	18/20	90	30	0.03	2			
12. 6-Morpholino- hexyl	50	16/20	80	20/20	100	20	0.03	1			
13 $\beta$ -n-Butylamino- ethyl	12.5	17/20	85	18/20	90	5	<4	50			
14 $\beta$ -Methyl- $\beta$ -nor- pholinopropyl	25	21/26	75	22/24	92	17	0.1	<2			
15 $\beta$ -( $\beta$ -Diethyl aminoethoxy)ethyl	50	13/19	63	18/20	90	22	0.00	<1			
16 $\beta$ -Dicyclohexyl aminoethyl	50‡	13/20	65	18/18	82	17	0.2				
17 $\beta$ -Di-n-butyl aminoethyl	50‡	16/20	80	19/20	95	15	0.15				
Benzhydryl Amines											
											
18. R = $\beta$ -Morpho- linoethyl	25	12/12	100	14/16	87		<2				
19 $\beta$ -Diethylamino- ethyl	25	12/16	75	19/24	79	4	<2				
20 $\gamma$ -Diethylamino- propyl	50	15/16	94	18/16	100	6	<1				
21 $\beta$ -Aminoethyl	50	14/16	87	15/16	94	7	<1				

Compound Nos. 10, 18, 20, 21 as dihydrochlorides Nos. 16 and 17 as bases all others excepting aminophylline as monohydrochlorides.

† P values from Fisher's Table. Values less than 0.05 indicative of significant difference.

‡ Figures in ( ) indicate LD-50 I.P. in guinea pigs.

§ Oral administration of base as 2 per cent aqueous gum acacia suspension 30 min. before subjecting animals to atomized histamine.

the three most potent benzhydryl ethers (12.5 mgm /kgm) induced no toxic responses and were capable of protecting all or a majority of animals from death on exposure to atomized histamine. In many animals dyspnea was the only symptom related to histamine treatment. No toxic effects of the drugs were observed. Oral administration of several of the most active benzhydryl ethers to guinea pigs conferred protection against atomized histamine.

The least active compounds studied, i.e., those with activity indices of 1 or 2, are equally as potent as aminophylline or papaverine. Several compounds were inactive at doses employed or else were not administered intraperitoneally because of their insolubility.

With the technique employed involving the inhalation of atomized histamine it would seem probable that any drug which reduced the minute volume of air breathed would lessen the severity of the experimental asthma. It is significant, therefore, that pretreatment of guinea pigs with two of the benzhydryl ethers proved an effective means of reducing the mortality due to intravenous administration of histamine. Compounds 1 and 2 were administered intraperitoneally in doses of 3.0 mgm /kgm thirty minutes before injecting histamine diphosphate (1.67 mgm /kgm) into the jugular vein. Whereas all of the 20 control animals which received saline placebos died within ten minutes after intravenous administration of histamine, the mortality rate was only 50 per cent in groups of 20 animals pretreated with either of the compounds.

**DISCUSSION** The technique described for selection and comparing the activity of compounds which have bronchodilator or anti-histamine activity has definite advantages such as rapidity of performance, simplicity, and reproducibility. Reduction in mortality as the criterion for drug effectiveness enhances the objectiveness and reliability of the method. Others (13, 26, 29-31) have depended almost wholly on more subjective data such as scores indicating the degree of asthma observed in animals exposed to atomized histamine. Information obtained from intact unanesthetized animals regarding bronchodilator or anti-histamine activity of drugs has definite value as an aid in the evaluation of such agents. In conjunction with other data, that obtained with the use of the method described should aid in the selection of specific histamine antagonists as well as musculotropic antispasmodics which have some selectivity of action on bronchiolar smooth muscle.

Presumably, the reduction in mortality and severity of symptoms noted after treatment with the benzhydryl ethers as well as with 929F and 1571F is referable, for the most part, to relief from the severe bronchoconstriction which is known to occur in guinea pigs following administration of histamine. Autopsies invariably revealed distended lungs in untreated control animals. This condition, which is indicative of severe bronchoconstriction, was not found in treated animals killed 4 to 10 minutes after exposure to atomized histamine solution. The depressor effects of histamine are diminished by 929F (37) and in view of the fact that the benzhydryl ethers (compounds 1 and 3, table 1) diminish the depressor effects of small doses of histamine and acetylcholine in dogs anesthetized with phenobarbital (unpublished data) it is possible that both types of compounds alleviate histamine shock by diminishing the vascular as well as the bronchiolar effects of

histamine. However, the predominant effect of atomized histamine in guinea pigs would probably be upon the bronchioles.

It is possible to draw several conclusions concerning the relation of chemical structure and anti-histamine activity based on the compounds reported in this study. A chain length of two carbon atoms is found in the five most active compounds (Nos 1-5). Compounds with longer or branched carbon chains are less active. This is apparent in comparing compound 3 with compounds 8, 12 and 14; however, the relative potency of Nos 6 and 7 opposes this view. An increase in chain length obtained by an oxygen interrupted chain (compound 15) also decreased the activity. The character of the substitution on the nitrogen atom is seen by comparing compounds 1-4 and 9 in which activity is in the order of tertiary amine > secondary amine > primary amine. In general, the data indicate that an increase in size of the group on the nitrogen atom leads to less active compounds in both the secondary and tertiary amines (compare compounds 4, 5 and 13, also 1, 6, 16 and 17). Compound 11 represents one example of substitution on the benzene rings of the benzhydryl group. On comparison with the analogous unsubstituted compound (No. 3) the 4,4'-dichlorobenzhydryl  $\beta$ -morpholinoethyl ether is seen to be much less active but less toxic. It is interesting that this compound in toxic doses produced depression in rats whereas the other ethers induced tremors and clonic convulsions. Compounds 18, 19, 20, and 21 are the amine analogues of compounds 3, 6, 7 and 9 in the ether series and it is evident that the replacement of the ether linkage by NH leads to inactive compounds.

The marked potency exhibited by these benzhydryl ethers in relation to effectiveness in alleviating the bronchoconstrictor effects of histamine administered as a mist or intravenously is of importance from several respects. Objectives in the selection of useful antispasmodics include the development of compounds with strong musculotropic activity (38) as indicated by their ability to antagonize substances such as histamine and barium chloride. With the exception of Demerol (31), gastro-intestinal or uterine antispasmodics now employed clinically are not noted for effectiveness in alleviating histamine-induced bronchoconstriction. The fact that benzhydryl ethers alleviate anaphylactic shock (39) as well as histamine shock supports the contention that histamine is probably intimately related to anaphylaxis. The marked anti-histamine activity of these synthetic antispasmodic compounds suggests experimental and clinical application in problems dealing with the role of histamine in gastric secretion, vasodilation, capillary permeability, pain mediation, smooth muscle spasm and various allergic syndromes.

#### SUMMARY

A method has been described for producing fatal bronchoconstriction in guinea pigs by administration of atomized histamine solution. The technique has been used to advantage in evaluating the ability of synthetic antispasmodics to alleviate the experimental asthma thus induced.

Three potent compounds selected from a series of benzhydryl alkamine ethers are  $\beta$ -dimethylaminoethyl  $\beta$ -piperidinoethyl and  $\beta$ -morpholinoethyl benzhy-

dryl ether. The activity of the first two compounds was demonstrated to be two to four times greater than that exhibited by the Fourneau histamine antagonists 929F and 1571F. Furthermore, they alleviated symptoms and diminished mortality in guinea pigs given intravenous injections of histamine. The activity of  $\beta$ -morpholinoethyl benzhydryl ether equalled that of 1571F. For each of the most active benzhydryl ethers the minimum dose administered intraperitoneally, which reduced the mortality rate a significant degree in guinea pigs subjected to atomized histamine, was only 1/30 to 1/60 of the intraperitoneal LD-50 in rats or guinea pigs.

The benzhydryl alkamine ethers are of special interest in view of their possible use in the study of physiological and pharmacological responses to histamine as well as the relationship of histamine to allergic and anaphylactic manifestations.

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# THE EFFECT OF LITHOSPERMUM RUDERALE ON THE ESTROUS CYCLE OF MICE<sup>1</sup>

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Lithospermum ruderale, a plant belonging to the Boraginaceae family, was used in the form of an infusion by American Indians in order to prevent conception (1). Experiments are herein reported in which the crude drug lowered the incidence of birth in mice by interference with estrous cycles. In addition, the drug caused a decreased rate of growth and a decrease in the weights of sex organs, thymus and pituitary gland.

**GENERAL METHODS** In all experiments a fluidextract was made of the ground plant, *Lithospermum ruderale*, with 50 per cent alcohol. The fluidextract was administered to animals by mixing with normal diet, ground Purina dog chow. In preparing a diet the desired amount of drug was mixed with part of the normal diet, this allowed to dry thoroughly and then brought up to final weight with addition of normal diet.

Diets were fed ad libitum, except where mentioned to the contrary, and a record of daily food intakes kept. When any spilling of food occurred it was taken into consideration in figuring food intakes. Animals were kept in individual cages in all experiments except those concerned with mating. The animals used were white mice raised in this laboratory from an inbred strain.

When mice were autopsied they were killed with ether, the organs carefully dissected out, immediately placed in a covered petri dish on moist, but not wet filter paper and then weighed in a weighing bottle on a chainomatic scale to a tenth of a milligram. The uterus plus vagina and seminal vesicles plus prostate were freed of their secretions before weighing. Statistical differences in organ weights were computed from the probable error of the mean.

Specimens of the plant were identified under the direction of Mr. B. Y. Morrison, principal horticulturist in charge of the Division of Plant Exploration and Introduction, Bureau of Plant Industry, Soils and Agricultural Engineering, United States Department of Agriculture.

**PART I MATING EXPERIMENTS** Young adult male and female mice were fed a 20 per cent *Lithospermum X* diet for ten days prior to and during the mating period of ten days. Three females were usually mated with one male. Of forty-four treated females fifteen, or 34 per cent, gave birth to young whereas twenty-eight, or 70 per cent, of forty control females littered. Thus the incidence of birth was reduced by 51 per cent in treated mice. The number of young per litter was not altered by the drug.

<sup>1</sup> This work was done in connection with the Indian Medicinal Plant study of the Bureau of Plant Industry, United States Department of Agriculture.

<sup>2</sup> This work was presented as a thesis to the Graduate School of the University of Minnesota in partial fulfillment of the degree of Doctor of Philosophy.

<sup>3</sup> *Lithospermum X* refers to drug picked in 1938, *Lithospermum Y* to drug picked in 1939 and percolated at a moderate rate and *Lithospermum Z* to drug picked in 1939 but percolated at a very slow rate. *Lithospermum X* was the most potent, *Lithospermum Y* relatively inactive and *Lithospermum Z* intermediate.

During the course of later experiments the drug appeared to be less active and therefore another mating experiment was conducted, using 20 per cent

Group A:

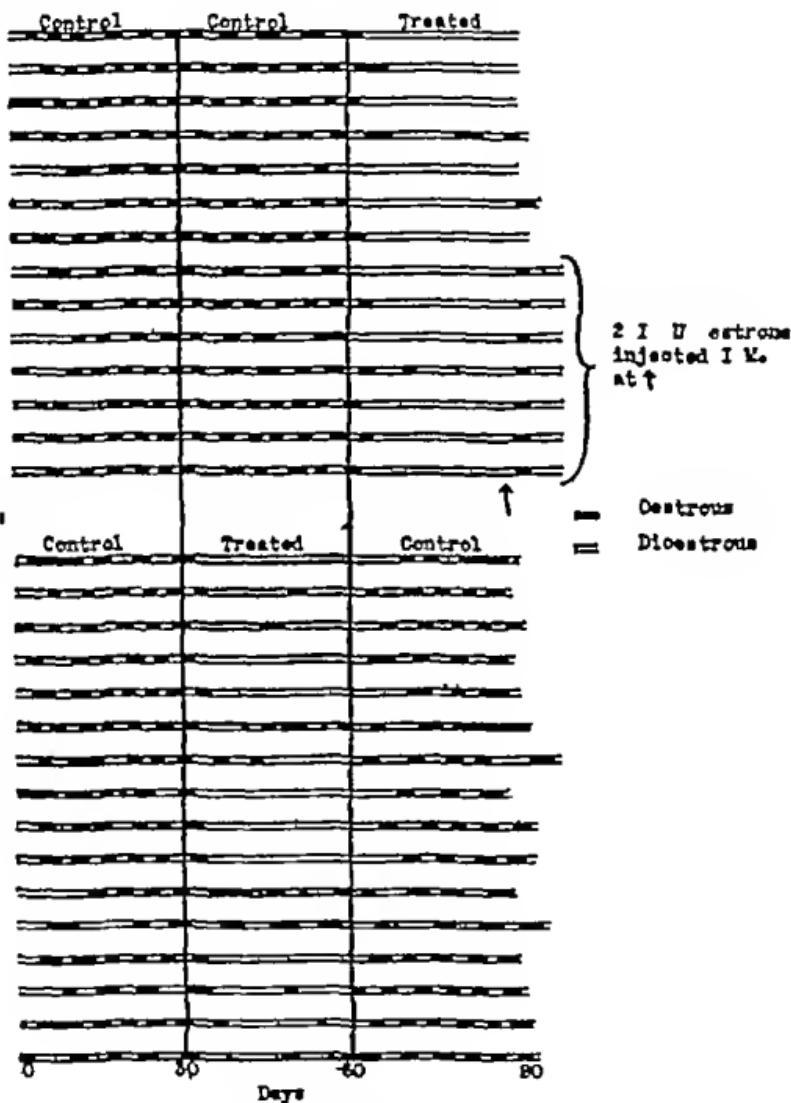


FIG. 1 ESTROUS CYCLES

Lithosperm Z. A decided decrease in drug potency was noted, the incidence of birth being reduced by only 12 per cent with the same concentration of drug.

PART II ESTROUS CYCLES. For these experiments female mice were separated from males at the time of weaning. When two to five months old the females

were placed in individual cages, fed normal diet, weighed every five days and their estrous cycles recorded. Vaginal smears were made daily from vaginal washings obtained with saline solution from a medicine dropper and examined microscopically. When mice were autopsied at the end of an experiment they were always killed during diestrus.

One experiment consisted of two groups, group A with fourteen mice and group B with sixteen mice. For thirty days both groups received normal diet, from thirty to sixty days group A continued on normal diet while group B was fed 40 per cent *Lithosperm Z*, from sixty to eighty-eight or more days the diets were shifted, group A receiving 40 per cent *Lithosperm Z* and group B returning to normal diet. At the end of the experiment the mice were autopsied. A record of the estrous cycles is shown in figure 1 and of the body weights and food intakes in figure 2.

Prolonged periods of diestrus occurred in most mice within a few days after being placed on treated diet. Twenty-one of the thirty mice showed no natural return of estrous during the treatment period, but after stopping drug administration estrous cycles returned quite promptly, in an average of 63 days.

TABLE 1

*The effect of *Lithosperm* on the weights of organs in adult female mice*

	NO. MICE	AVERAGE WEIGHT (GRAMS)						
		Ovaries	Uterus + vagina	Adrenals	Thyroid	Thymus	Pituitary	Kidneys
Group B, control	16	0.0176	0.1019	0.0092	0.0188	0.0291	0.0020	0.3889
Group A, treated								
No estrone	7	0.0162	0.0716*	0.0078	0.0170	0.0156*	0.0017	0.3603
After estrone	7	0.0113	0.0461	0.0063	0.0175	0.0238	0.0016	0.3249

\* Denotes statistical difference when calculated as per cent of body weight.

(group B) Seven mice in group A received, near the end of the experiment, a single intramuscular injection of 2 I.U. of estrone<sup>4</sup> in sesame oil while being maintained on the treated diet. This produced estrous in all mice in an average of forty-nine hours.

In table 1 are recorded the weights of organs at autopsy. Statistical decreases were observed in the weights of the uterus plus vagina and of the thymus of treated animals, the uterus plus vagina of group A receiving no estrone being decreased by 29 per cent, the thymus by over 46 per cent. The kidneys of mice in group A showed some decrease in weight compared with group B. This decrease was not significant but suggests the lack of a pro-renal effect of the drug.

Figure 2 indicates that a decrease in body weight in the presence of an increase in food consumption is characteristic of treated mice. The average food intake per mouse per day for control mice (both groups) was 4.5 grams and for treated mice (both groups) was 6.4 grams, an increase of 42 per cent for

<sup>4</sup> A single intramuscular injection of 2 I.U. of estrone given to eight ovariectomized female mice caused estrous in an average of forty-four hours, one I.U. in an average of fifty-nine hours.

treated animals. One hundred grams of 40 per cent Lithosperm Z diet contained only 11 grams of dried drug so the fact that part of the diet was not normal food cannot account for all the increased food intake.

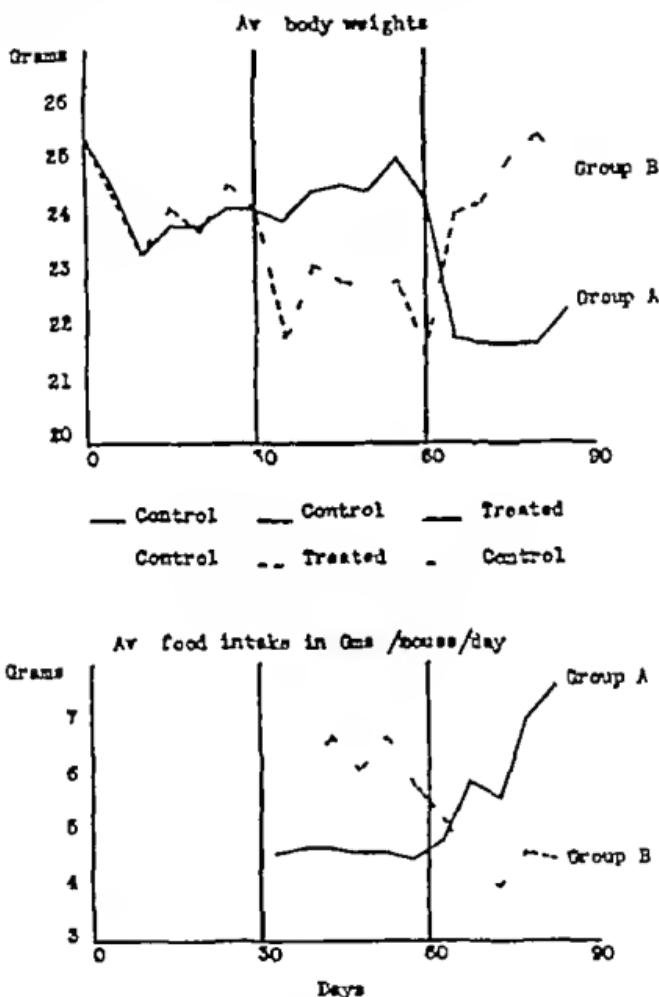


FIG. 2 AVERAGE BODY WEIGHTS AND FOOD INTAKES FOR MICE IN FIGURE 1

Stopping drug administration caused a quick return to normal of estrous cycles, body weight and food intake. Thus indicates that effective concentrations of the drug were rapidly eliminated or destroyed in the body.

Another experiment in this group included fourteen mice seven control and seven treated (40 per cent Lithosperm Z diet). The estrous cycles are recorded in figure 3, the body weights and food and water intakes in figure 4. Again Lithosperm caused prolonged periods of diestrous. After forty-six days of treatment follicle stimulating hormone (FSH) in the form of pituitary syn-

gist<sup>5</sup> was injected subcutaneously twice a day for seven days, the total dose being 0.35 mgm. This dose was ineffective in inducing estrous. However the higher dose of 3 mgm injected over nine days caused the development of estrous, thus indicating that the ovaries were capable of being stimulated by this gonadotropic hormone. Lithosperm administration was continued during treatment with FSH.

Mice treated with Lithosperm again showed the characteristic loss of weight in the presence of an increased food intake. Treated mice lost approximately 10 per cent of their body weight during the first ten days of treatment, then more or less plateaued at this level until about the eightieth day when they began to gain and at a hundred days again equaled the controls in weight. Daily food intake of treated mice was approximately 40 per cent greater than

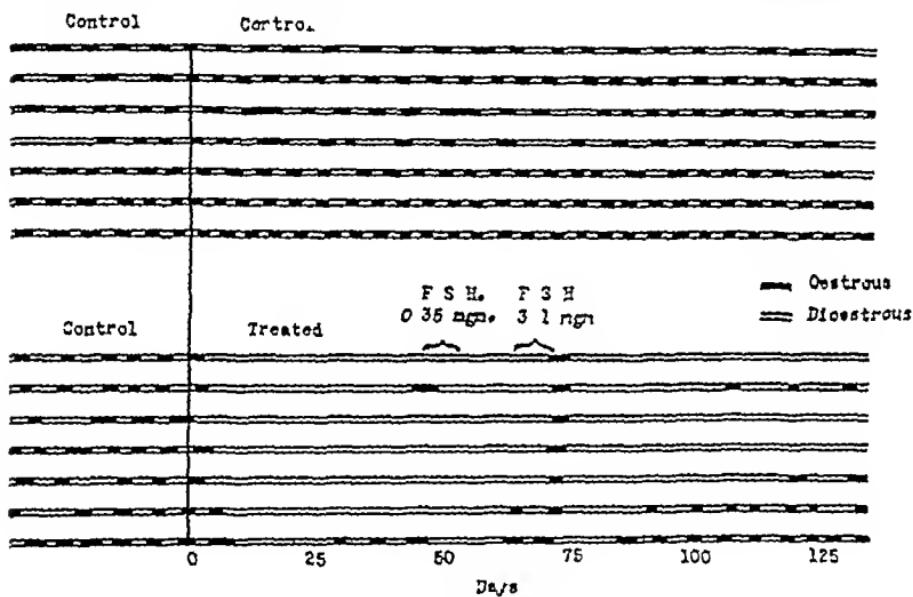


FIG. 3 ESTROUS CYCLES

that of controls. Water intake of treated mice was markedly increased at first, being 103 per cent greater than the controls the first twenty days, but decreased to only 32 per cent greater than controls during the last twenty-five days. Correlating water intake with food consumption showed that during the first part of the experiment 2.15 cc of water were taken by treated mice for every gram of food, 1.45 cc of water per gram of food by controls, but by the end of the experiment both groups were drinking 1.5 cc of water per gram

<sup>5</sup>The pituitary synergist was kindly furnished by Dr. D. K. Kitchen of Parke, Davis and Co., Detroit, Mich. When injected subcutaneously twice a day for three days in female mice, twenty-one days old, in a total dose of 0.30 mgm. this preparation caused an increase of 240 per cent in the weights of uterus plus vagina and of 30 per cent in the weights of ovaries, as noted at autopsy occurring seventy two hours after the first injection.

of food. Water intake of treated mice decreased about the same time that their body weights increased. No records of urine output were made but sawdust under the cages was noted to be definitely wetter during periods of increased water intake.

PART III EFFECTS IN MICE TWENTY-ONE DAYS OF AGE. A study of the effect of Lithosperm on the growth of various organs was made in litter mate mice

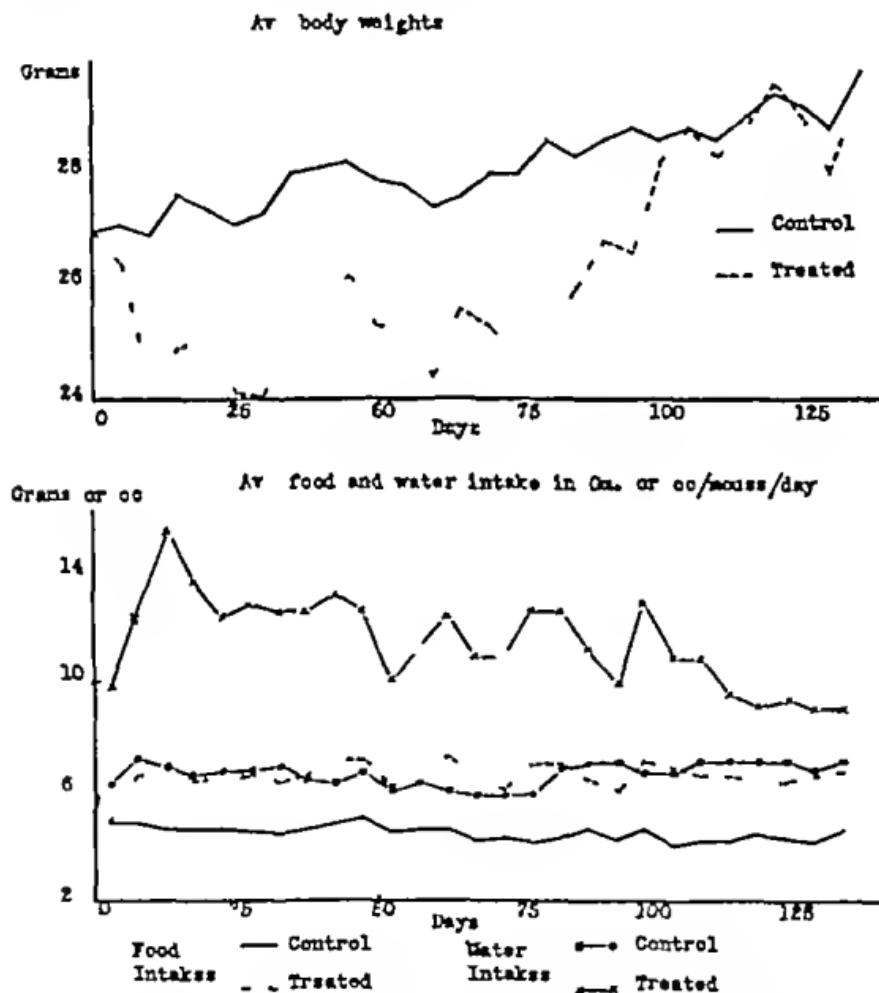


FIG. 4 AVERAGE BODY WEIGHTS AND FOOD INTAKES FOR MICE IN FIG. 3

twenty-one days of age. Litters were restricted to six young in order to obtain a more uniform weight of each.

Twenty five female mice were fed 40 per cent Lithosperm Z for ten days and then autopsied. Twenty five female litter mates served as controls. Decreases in the weights of ovaries (37 per cent) uterus plus vagina (51 per cent),

thymus gland (62 per cent) and pituitary gland (40 per cent) were observed in treated mice with a decrease in body growth of 25 per cent (figure 5). The differences in the weights of these organs were statistically significant when calculated as per cent of body weight. In addition twenty-five untreated stock mice weighing the same as treated mice at time of autopsy were killed. Their average age was twenty-four days, seven days younger than the treated and control mice, yet their ovaries, uterus plus vagina and thymus glands weighed more than those of treated mice. A fourth group of mice consisted of ten stock females autopsied at twenty-one days of age in order to indicate the approximate weights of organs of treated and control mice at the beginning of the experiment. From the weights obtained in these four groups of animals growth curves of the various organs were drawn (figure 5). These graphs clearly indicate that the factors responsible for growth of ovaries, uterus plus vagina, thymus and pituitary gland between twenty-one and thirty-one days of age were not functioning normally in treated mice.

A similar experiment was conducted with 40 per cent *Lithosperm Z* for ten days in fifteen male mice twenty-one days of age. Decreases in the rate of body growth of 34 per cent, in the weight of testes of 28 per cent, of seminal vesicles plus prostate of 66 per cent, of thymus of 67 per cent and of pituitary gland of 45 per cent were noted in treated mice compared with their fifteen litter mate controls. The decrease in weight of the seminal vesicles plus prostate and the thymus of treated animals was significant when calculated as per cent of body weight. Fifteen stock mice weighing the same as treated mice at autopsy whose average age was 24 days and fifteen stock mice twenty-one days of age were also autopsied. Figure 6 shows that the testes of treated animals grew at a rate similar to body growth, but seminal vesicles plus prostate were inhibited to a greater extent, as was the thymus, which in fact regressed to a marked degree. The results also show a complete lack of growth of the pituitary gland in the treated group. However the difference in weight between the pituitary glands of treated and control animals was not statistically significant if calculated as per cent of body weight.

Results of experiments not reported here suggest that *Lithosperm* is more active in immature female mice than in immature males for relatively inactive preparations (*Lithosperm Y*) caused decreases in the weights of sex organs of female but not of male mice.

The weights of adrenal and thyroid glands were always recorded in autopsied mice but no changes which were significant as per cent of body weight were noted in either immature or adult treated mice.

**PART IV INANITION** That inanition causes a decrease in the growth of sexual organs is well known (2). That inanition could be the cause of the effects of *Lithosperm* seemed unlikely inasmuch as treated mice consumed more food in all experiments than their controls and showed no apparent increase in fecal material. However to investigate this possibility the following experiment was conducted.

Mice, twenty-one days of age, were divided into three groups, controls,

those on 40 per cent Lithosperm Z diet and restricted controls, the latter receiving normal diet but in amounts only sufficient to allow for increases in body weight comparable to those of treated animals. Litter mates were used as frequently as possible. After ten days the mice were autopsied. Two such experiments were performed, one with female and one with male mice. Table

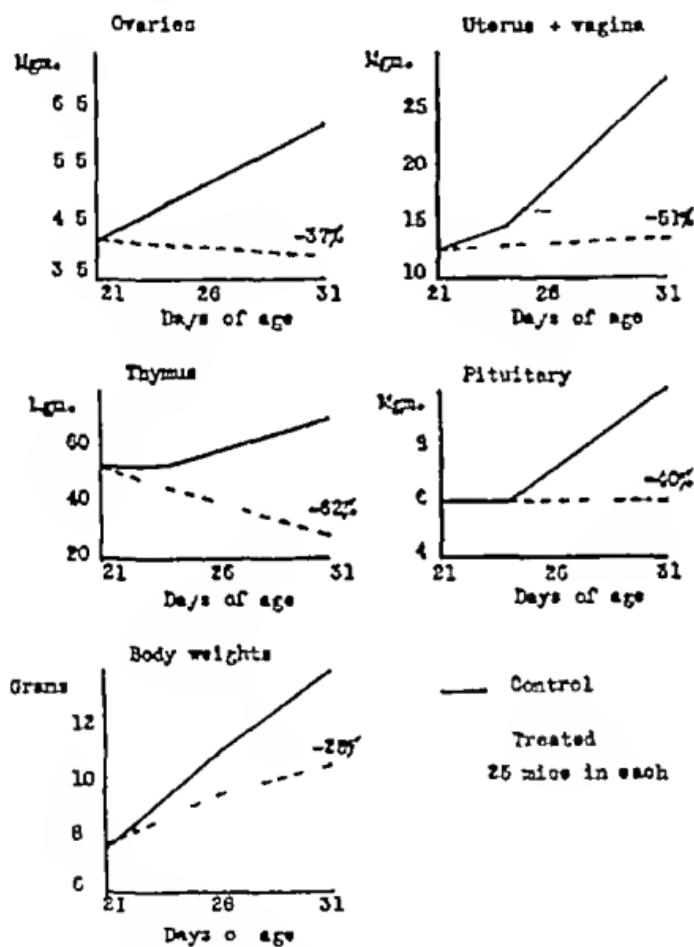


FIG. 5 ORGAN GROWTH RATES IN IMMATURE FEMALE MICE

2 shows that restricted controls gained weight at the same rate as treated mice and that the degree of inanition in the restricted controls was sufficient to cause a decrease in the growth rate of sex organs. However the growth of uterus plus vagina and seminal vesicles plus prostate in treated mice was significantly less than in the restricted controls.

In female mice the body weight of restricted controls compared with normal

controls, was depressed by 18 per cent at time of autopsy, ovaries by 13 per cent and uterus plus vagina by 38 per cent. The body weight of treated females, compared with normal controls, was decreased by 20 per cent, ovaries by 22 per cent and uterus plus vagina by 59 per cent. The decrease in weight of the

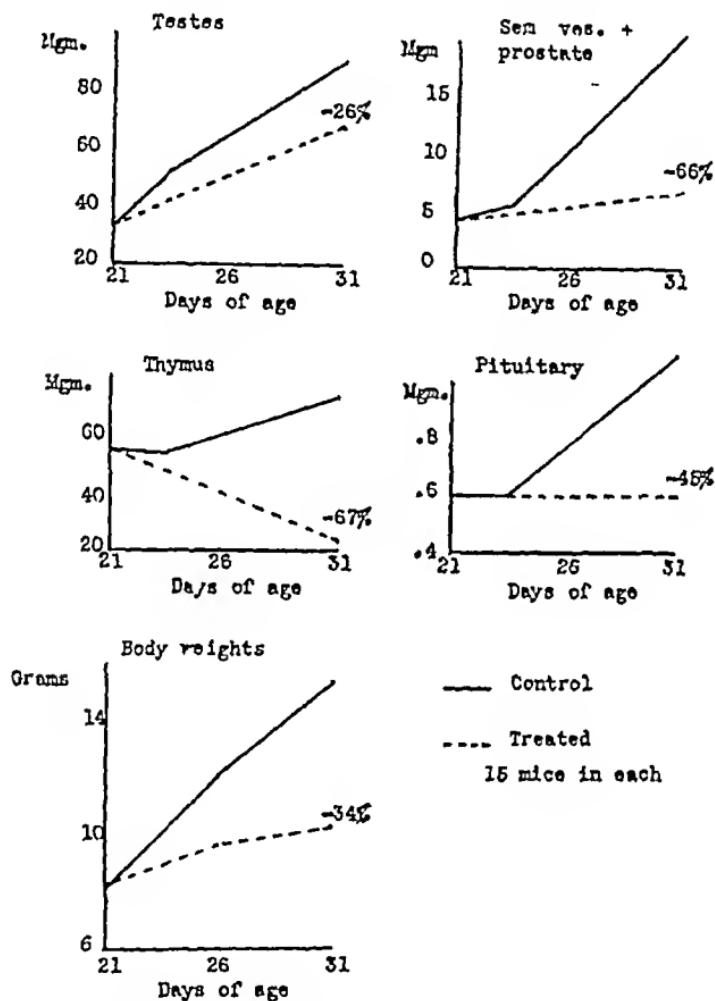


FIG. 6 ORGAN GROWTH RATES IN IMMATURE MALE MICE

uterus plus vagina was significantly greater in treated animals than in the restricted controls.

In male mice the body weight of restricted controls was decreased by 29 per cent compared with normal controls, the testes by 10 per cent and the seminal vesicles plus prostate by 50 per cent. The body weight of treated mice, compared with normal controls, was reduced by 30 per cent, the testes by 20 per cent and the seminal vesicles plus prostate by 68 per cent. This retardation of

growth of the seminal vesicles plus prostate was significantly greater in treated mice than in restricted controls.

These results indicate, therefore, that inanition, *per se*, cannot account entirely for the decrease in growth of sex organs observed in mice treated with Lithosperm.

TABLE 2  
Comparison of the effects of *Lithosperm* and inanition  
I Average body weights and food and water intakes in grams or cc

	NO. MICE	AV. BODY WT.			AV. FOOD INTAKE		AV. WATER INTAKE	
		At days			During days		During days	
		0	5	10	0-5	5-10	0-5	5-10
Female mice								
Control	15	8.7	11.6	14.8	2.9	3.7	4.1	5.0
Treated	16	8.6	9.9	11.9	3.4	4.2	4.7	6.0
Restricted control	17	8.8	10.3	12.2	2.3	2.8	3.8	4.4
Male mice								
Control	16	8.9	12.7	17.1	3.1	4.1	4.8	6.8
Treated	18	9.1	10.6	12.0	8.6	4.2	4.3	6.1
Restricted control	19	8.9	10.7	12.1	2.5	2.6	4.7	5.7

II Organ weights at time of autopsy (10 days)

	OVARIES OR TESTES	UT + VAGINA OR S.V. + PROSTATE		ANTERNA		THYROID		TESTES		PITUITARY	
		Gram	%	Gram	%	Gram	%	Gram	%	Gram	%
Female mice											
Control	0.0068	0.0450	0.0337	0.2280	0.0410	0.0280	0.0151	0.1020	0.0799	0.5390	0.0009
Treated	0.0049	0.0410	0.0142	0.1190	0.0350	0.0290	0.0129	0.1090	0.0554	0.4650	0.0006
Restricted control	0.0056	0.0460	0.0208	0.1700	0.0370	0.0300	0.0157	0.1290	0.0541	0.4130	0.0008
Male mice											
Control	0.0968	0.5660	0.0278	0.1610	0.0033	0.0190	0.0158	0.0930	0.0801	0.4680	0.0011
Treated	0.0772	0.6430	0.0091	0.0760	0.0030	0.0250	0.0182	0.1100	0.0376	0.3180	0.0006
Restricted control	0.0689	0.7180	0.0189	0.1150	0.0032	0.0260	0.0156	0.1280	0.0372	0.3070	0.0007

\* Denotes statistical difference when compared with restricted controls.

PART V BASAL METABOLIC RATE. Inasmuch as thyroid activity may affect estrous cycles and growth, the metabolic rate of control and treated mice was compared. The apparatus used was similar to one designed by Tainter (3). Food was withdrawn four to six hours before testing and an intraperitoneal injection of 0.01 mgm. pentobarbital per gram of body weight was given one

hour before testing to produce sedation. Activities of the animals were more constant with this latter procedure. A series of twenty readings were taken for each animal, measuring with a stop watch the number of seconds required to consume 2 cc of oxygen. The longest of these readings was taken to represent basal conditions. The number of cc of oxygen used per minute per 100 sq cm of body surface was then calculated for standard pressure and temperature.

No difference was observed between treated and control mice, both groups consuming an average of 1.13 cc of oxygen at S.T.P. per minute per 100 sq cm of body surface. The range for the seven control mice was 0.75 to 1.50 cc of oxygen and for seven treated mice was 0.69 to 1.44 cc of oxygen. Thus *Lithosperm* did not seem to alter the basal metabolic rate of mice. However this test was made on the mice represented in figures 3 and 4 near the end of that experiment, at which time the treated mice had gained weight and some were showing a return of estrous cycles. Therefore it is possible that differences in metabolic rates might occur in mice under treatment for a shorter period of time. It has not been possible to repeat this experiment or to do any further research with *Lithosperm* for there has been no more drug available.

**DISCUSSION** The prolonged periods of diestrous and the decrease in weight of the uterus plus vagina produced by *Lithosperm* in female mice indicate an inhibition in the formation or secretion of estrogenic hormone or an interference with its action on the uterus and vagina. Inasmuch as estrous was initiated by injected estrone and the ovarian weights were decreased in treated animals, a decrease in the formation of this hormone is probable. A deficiency in estrogenic hormone may be due in turn to an inhibition in the formation or secretion of follicle stimulating or luteinizing hormone, or to some interference with their actions on the ovaries. Injections of FSH (which may have contained small amounts of LH) into mice in prolonged states of diestrous were effective in producing estrous, showing that the ovaries were capable of responding to gonadotropic stimulation by secreting estrogenic hormone. Therefore the occurrence of a deficiency in the formation or secretion of FSH or LH caused by the drug is indicated. In addition, *Lithosperm* caused reductions in the weights of pituitary glands, suggesting a decrease in the formation of, rather than just secretion of, gonadotropic hormone.

The decrease in weight of the seminal vesicles plus prostate caused by *Lithosperm* in immature male mice demonstrates a decrease in androgenic activity. No experiments administering androgenic or gonadotropic hormones to male animals were conducted, but the decreased weight of the pituitary gland points again to an effect of the drug on this gland. The slight decrease in weight of testes but much greater decrease in seminal vesicles plus prostate suggests that luteinizing hormone may have been inhibited to a greater extent than follicle stimulating hormone.

Although no direct assays of gonadotropic activity of pituitary glands have been made, the above experiments lead to the conclusion that gonadotropic activity is inhibited by the crude drug *Lithosperm*.

Estrogens and androgens are both known to inhibit gonadotropic activity (4) but it is apparent from the above experiments that *Lithosperm* does not

exert an estrogenic or androgenic action. Progesterone also inhibits gonadotropic activity, suppressing the release of LH from the pituitary gland (5 6) and causing inhibitions in estrous cycles (7, 8). However other effects of progesterone include an increase in the weights of seminal vesicles in the presence of testicular tissue (9 10 11) a decrease in adrenal weights and an hypertrophy of the uterus (12, 13), none of which were noted in mice treated with Lithosperm. Adrenal cortical preparations in the form of desoxycorticosterone acetate may cause reduction in the weights of testes (14) but other effects include enlargement of the kidneys (15) and a decrease of adrenal weights (16). Lithosperm seemed to exert no renotropic action and failed to produce significant changes in the weights of adrenal glands. The thyroid hormone also is concerned in gonadotropic functions, inhibiting the action of FSH on the ovaries (17, 18). That Lithosperm acts via the thyroid gland is improbable, in view of the results obtained on metabolic activity, but has not been definitely ruled out. Also the direct comparison of the effects of inanition and of Lithosperm indicates that the action of this drug was not due to inanition *per se*. Therefore the conclusion drawn is that Lithosperm causes a depression of gonadotropic activity in some way heretofore unknown. Its action is probably a direct one on the pituitary gland for the drug caused a decrease in the weight of this organ.

The loss of weight in adult mice and the decreased rate of growth in young animals in the presence of increased food intakes point to the possibility of some metabolic change occurring as the result of treatment. The anterior pituitary gland appears to possess hormones which affect all classes of foodstuffs (19 20) and therefore a drug which alters the function of this gland might produce changes in metabolism. Also androgens and estrogens seem capable of altering metabolic activities having been shown (21, 22 23) to cause retention of nitrogen with increase in body weight on a constant food intake and without change in metabolic rate. In addition these hormones produced decreases in the urinary excretion of electrolytes and a reduction in urinary volume. On this basis the opposite effect, a deficiency of estrogens or androgens might be accompanied by a loss of weight without a decrease in food consumption or in increase in metabolic rate as noted in mice treated with Lithosperm. Such a deficiency might also be expected to cause an increase in the urinary excretion of electrolytes and an increase in urinary volume. Of interest in this connection is figure 4, showing that water taken in *ad libitum*, by treated animals was roughly inversely proportional to their body weight. During the acute loss of weight the water intake was markedly increased when the weights tended to plateau so did water consumption and when body weight increased near the end of the experiment the excessive intake of water decreased. Urinary output was not recorded, nor were any urinary analyses made.

All treated mice, both immature and mature seemed to be in a healthy condition as judged by subjective signs such as activity and appearance of fur.

#### SUMMARY

1. Fluidextract of *Lithospermum ruderale* decreased the incidence of birth in mice.

2 Adult female mice with previously regular estrous cycles developed prolonged periods of diestrous when treated with this drug. Injections of estrone or of gonadotropic hormone were capable of inducing estrous in treated animals.

3 In immature male and female mice treatment with Lithosperm caused decreases in the weights of the sex organs, thymus and pituitary gland and a retardation in body growth.

4 The inhibition in the growth of sex organs was not due to inanition per se.

5 Discussion of the results obtained leads to the conclusion that Lithosperm probably acts directly on the pituitary gland causing an inhibition in the formation of gonadotropic hormone. The effects of this drug do not seem to be duplicated by other known compounds.

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# ON THE COMBINATION OF SOME CARDIO-ACTIVE GLYCOSIDES WITH SERUM PROTEINS

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Lendle et al (1) and Fawaz and Farah (2) have demonstrated the combination of digitoxin with rabbit serum albumin. The latter authors have shown that it is this combination which is responsible for the protection of the isolated frog heart against digitoxin. Beef-serum (Santesson and Strindberg (3)) and frog serum (Hoekstra (4)) do not give any appreciable protection against digitoxin. In our laboratory, horse serum has shown protective action but was found to be much weaker than rabbit serum. Lendle et al (1) used *in vitro* methods to determine the combination of a number of cardio-active glycosides with serum albumins and found that the serum albumins of man, rabbit, and horse have similar powers of combining with digitoxin.

Clark (5) and Oppenheimer (6) report that rabbit serum does not protect against strophanthin, but Lendle et al. (1) have found that strophanthin combines with serum albumin *in vitro*. These observations of Lendle et al. (1) regarding the power of serum albumins of various species to combine with cardio-active glycosides are incompatible with the earlier results mentioned above. An explanation for this discrepancy might lie in the variable albumin-globulin ratios of various species, since Lendle et al. were working with isolated serum albumins while the other workers were working with total serum. Another possible source of discrepancy is that when measuring serum protein-glycoside combination *in vitro* one might be dealing with a mechanism which differs from that which is responsible for the protection of the isolated frog heart against cardio-active glycosides.

The object of this paper is to clarify and harmonize, if possible, some of these discrepancies. First, we compared the protective action of serum proteins of different species with the *in vitro* combining power of these proteins with digitoxin. Second we determined the protective action of rabbit serum against a number of cardio-active glycosides as compared with the power of combining with these respective glycosides. The third experiment dealt with the protective action of rabbit serum against a number of cardiac aglucones. Since the quantity of aglucones available was very limited, we were unable to carry out determinations of combining-power *in vitro*.

In the latter part of this paper we report briefly on some factors which influence the albumin-digitoxin combination. We also describe a number of experiments performed on the intact anesthetized cat and dog and on the heart-lung preparation (H.L.P.) of the dog to see if the combination of digitoxin with serum proteins modifies in any way the lethal dose (L.D.) of this glycoside.

MATERIALS AND METHODS Serum and Serum Proteins were prepared by

methods previously described (2) We have prepared sera of man, rabbit, dog, horse, cat, frog, rat, and pigeon Serum albumins and globulins were prepared from the sera of man, rabbit, dog, horse, and cat These serum proteins were purified by three reprecipitations, but no attempt was made to prepare crystalline material, since the protective action of crystalline rabbit serum albumin was not significantly different from that of more crude preparations (2)

The cardio-active glycosides employed were

Crystalline digitoxin, (Hoffmann La Roche)<sup>1</sup>

Crystalline digilanid, (Sandoz—a mixture of the three genuine Lanata glycosides)<sup>2</sup>

Crystalline g-strophanthin Thoms, (E Merck, Darmstadt)

Amorphous k-strophanthin U S P, (Merck & Co Inc, Rahway, N J)

Crystalline foliandrin, (Teva)<sup>3</sup>

The last mentioned glycoside was prepared from *Nerium oleander* leaf (7), and has physical and biological properties which are similar to those reported for the folinerin of Flury and Neumann (8)

The aglucones of k-strophanthin, digitoxin and foliandrin were prepared by the methods of Jacobs and Heidelberger (9), Cloetta (10) and Neumann (11) respectively The aglucone of foliandrin was a crystalline material with the same physical properties as the aglucone of folinerin (11)

Protection was determined by exposing the Straub isolated heart of *Rana temporaria* to varying known concentrations of cardio-active glycosides in the presence or absence of serum proteins At least six hearts were exposed to each test solution After 60-70 minutes the experiment was terminated and the effect recorded as (+++), (++) , (+), (±) and (-)<sup>4</sup> Each experiment was performed on a uniform batch of frogs during a period not exceeding ten days All specimens of serum proteins used in studying protection were tested alone on the isolated frog heart and any toxic specimens discarded

Combining power was determined by dialyzing solutions of the glycosides dissolved in serum against an equal volume of Locke's solution for 48-72 hours<sup>5</sup> The concentrations of glycoside in the dialysates were determined by the Balyat alkaline picric acid reaction as used by Lendle and Pusch (12) Control experiments showed that equilibrium was reached between inside and outside fluids within 48-70 hours Since the presence of serum in the inside fluid would lower the glycoside concentration in the dialysate to less than the theoretical quantity, the difference between theoretical and actual concentration of glycoside in the

<sup>1</sup> Kindly supplied by Hoffmann La Roche, Basel, Switzerland

<sup>2</sup> Kindly supplied by Sandoz, Basel, Switzerland

<sup>3</sup> Kindly supplied by Teva, Middle East Pharmaceutical and Chemical Works Co Ltd, Jerusalem, Palestine

<sup>4</sup> (+++) No effect (complete protection)

(++) Reduction in amplitude up to 30%

(+) Reduction in amplitude from 31-60%

(±) Reduction in amplitude from 61-90% or the appearance of irregularities

(-) Complete systolic standstill

<sup>5</sup> For these experiments we have used cellophane dialyzing bags supplied by the Visking Corporation, Chicago, U S A

dialysate was assumed to be due to combination with serum protein and was recorded as *combined*. A series of experiments was performed to see if there was any loss of activity of the glycoside during the period of dialysis. Since it was possible that the colorimetric determination might not coincide with biological activity we compared these two methods simultaneously as an essential control experiment. Our results with digitoxin, digilanid, folandrin g and L-strophanthin show that at the end of the period of dialysis there was equilibrium between inside and outside solution and that there was no destruction of glycoside which could be detected either by the biological or colorimetric methods of assay.

The Balyat reaction is a useful method for determining concentrations of certain cardio-active glycosides. It should be remembered, however, that different batches of a given substance may give different color intensities. We have tested two preparations of digitoxin, one supplied by Merck, the other by Hoffmann La Roche. Both digitoxin preparations had the same biological activity but had divergent color intensities. However, if one uses a uniform specimen of glycoside for a given series of experiments, the results with the Balyat reaction are consistent. In carrying out the Balyat reaction, the glycoside solution is mixed with the alkaline picrate and placed in a constant water bath at 36°C. Fifteen minutes later the solution is transferred to the cup of a Duboscq colorimeter and immediately matched against 0.344 per cent potassium dichromate solution. Using this method we have been able to determine accurately glycoside concentrations as small as 1:200,000. With more dilute solutions the error is rather high. Colorimeter readings for known concentrations of glycoside were plotted against concentration and the resulting curve was used for determining unknown solutions. All solutions which became turbid on the addition of picric acid were discarded. Determinations were always made in duplicate and averaged. The picric acid used was first carefully purified by the method of Folin (13).

*Determination of the Lethal Dose of Digitoxin for Intact Cats and Dogs* was performed on anesthetized animals (0.04 gms Nembutal Abbott/kg). A 1:50,000 solution of digitoxin Hoffmann La Roche in saline or 5% rabbit serum protein was infused into the femoral vein. The rate of infusion was regulated in such a way that death would occur within 40-60 minutes. All animals were given artificial respiration with a Palmar respiration pump. The variations in body weight were between 1.8-3.5 and 4.0-7.6 kg for cats and dogs respectively.

H.L.P. were prepared on dogs anesthetized with chloralose (90 mg/kg) using the method of Starling. Only hearts weighing between 45.5 and 67 gms were used. A 1:50,000 solution of digitoxin, in saline or 5% serum protein was infused at a slow constant rate into the venous end of the circulation. Inflow per gram heart per minute varied between 0.035 to 0.084 micrograms of digitoxin. The end point was reached between 88-134 minutes. Temperature, blood volume, resistance and output were the same for all the experiments performed.

**RESULTS I** Table 1 gives the results of one of three similar experiments on the protective action of serum and serum proteins of the various species of animals studied. It is apparent that there are detectable species differences in protection both with serum and serum albumin. Rabbit serum and serum albumin gave the

highest protection, dog serum gave less protection, and horse serum and serum albumin were the weakest. Simultaneously, combining power was determined on the same serum protein specimens using 0.5 percent protein and 1/30,000 digitoxin. These results are given in the last column of table 1. Rabbit serum shows the highest combining power and horse serum protein the lowest, while dog serum protein shows an intermediate degree of combining power. The globulin fractions of sera of rabbit, dog, and horse showed neither protection nor combination with digitoxin and have not been included in table 1. Comparison of the

TABLE 1

*Comparison of the protective ability with the combining power of serum and serum albumins of various species with digitoxin Hoffmann La Roche*

	NUMBER OF HEARTS USED	NUMBER OF HEARTS SHOWING EFFECT					TIME IN MINUTES FOR MAXIMAL EFFECT	NUMBER OF HEARTS SHOWING RECOVERY WITHIN 60 MIN	COMBINING POWER OF SERUM PROTEIN AT 0.5% PROTEIN AND 1/30,000 DIGITOXIN MG DIGITOXIN PER GM. PROTEIN
		+++	++	+	+	-			
Digitoxin 1/100,000 in Locke's solution	8	0	0	0	1	7	12	0	
Digitoxin 1/100,000 in 1 per cent rabbit serum protein	8	0	4	4	0	0	33	8	2.440
Digitoxin 1/100,000 in 0.5 per cent rabbit serum albumin	8	0	5	3	0	0	38	8	3.875
Digitoxin 1/100,000 in 1 per cent dog serum protein	8	0	1	6	1	0	24	7	1.034
Digitoxin 1/100,000 in 0.5 per cent dog serum albumin	8	0	2	5	1	0	25	6	2.070
Digitoxin 1/100,000 in 1 per cent horse serum protein	8	0	0	0	5	3	18	2	0.585
Digitoxin 1/100,000 in 0.5 per cent horse serum albumin	8	0	0	0	6	2	20	2	0.065
Digitoxin 1/100,000 in 1 per cent frog serum protein	6	0	0	0	3	3	18	2	0.855

protective action of a given serum protein solution with its combining power for digitoxin shows a close positive correlation between the two. In view of the fact that the species differences in combining power and protective ability are also apparent in the serum albumin fractions, it is not possible to explain Lendle's findings on the basis of differences in the albumin-globulin ratios.

Table 1 includes results obtained with frog serum. This experiment was performed at a different season and thus is only qualitatively comparable with the other data. However, on the basis of both the protection tests and the in-

*vitro* procedure we conclude that frog serum shows a definite combination with digitoxin. This finding is contrary to those of Hoekstra (4) but confirms the observations of Brücke (14).

Table 2 summarises the significant findings in a number of experiments carried out to determine the digitoxin combining power of serum proteins. These experiments, which do not include protection tests were extended to include certain additional species. In respect to combining power both serum and serum albumin of man and rabbit gave the highest values. The serum proteins of cat, rat, dog and pigeon were intermediate in combining power, while serum and serum albumin of the horse gave the lowest values. Frog serum like that of the horse, showed little ability to combine with digitoxin, the combining power of frog serum

TABLE 2

*Ability of serum and serum albumin of different species to combine with digitoxin*

Values are mg of digitoxin per gram protein. Determinations were made with 1500 0.5% protein and 1/30,000 digitoxin in Locke's solution dialyzed against an equal volume of Locke's solution for 65-70 hours. Letters with figures are initials of individual human blood donors.

HUMAN		CAT		DOG		HORSE		RABBIT		PIGEON		RAT	FOX
Serum	Albumin	Serum	Albumin	Serum	Albumin	Serum	Albumin	Serum	Albumin	Serum	Albumin	Serum	Serum
3 815 (F)	3 540 (F)	1 906	2 866	1 436	2 070	0 406	0 766	2 440	3 875	1 309	1 133	0 855	
2 500 (A)	2 855 (A)	2 000	2 895	1 034	1 500	0 835	0 965	3 026	3 466	1 472	1 650	0 920	
3 242 (B)	3 480 (F)	1 680	2 785	1 186	2 1750	0 785	0 600	2 010	3 330			1 790	1 050
2 655 (A)	2 510 (A)	1 455		1 533	2 660	0 930	0 808	2 106	3 425				
3 200 (F)	3 510 (B)			1 225	2 333	0 828		2 466	3 550				
				1 080		0 800		3 006	3 420				
								2 200					
								2 706					
								2 492					
								1 800					
Average	2 932	3 179	1 753	2 849	1 249	2 2080	0 743	0 784	2 434	3 511	1 421	1 524	0 941

albumin was not determined. It is particularly interesting that in the case of human serum and serum albumin, appreciable differences in digitoxin-combining power were shown by sera from different individuals. These differences were constant for specimens of serum and serum albumin prepared at different times (table 2).

The values of the combining power discussed above were determined with solutions of 0.5 per cent protein and 1/30,000 digitoxin. We have demonstrated that the differences described above are also apparent when the protein concentration is varied (fig 1). On the other hand when the protein concentration was kept constant and the amount of digitoxin varied the results were different. At low digitoxin concentrations the species differences in combining power of serum albumin were clearly seen, but at concentrations above 1/10,000 these differences

were much less apparent. The combining power of rabbit and horse serum albumin were about equal when a 1:7,500 digitoxin concentration was used (fig 2). Goigner and Pauly (15), while studying the combination of silver and protein, noted that if combining power was calculated on the basis of per gram protein, there was a progressive reduction of combining power as the concentration of

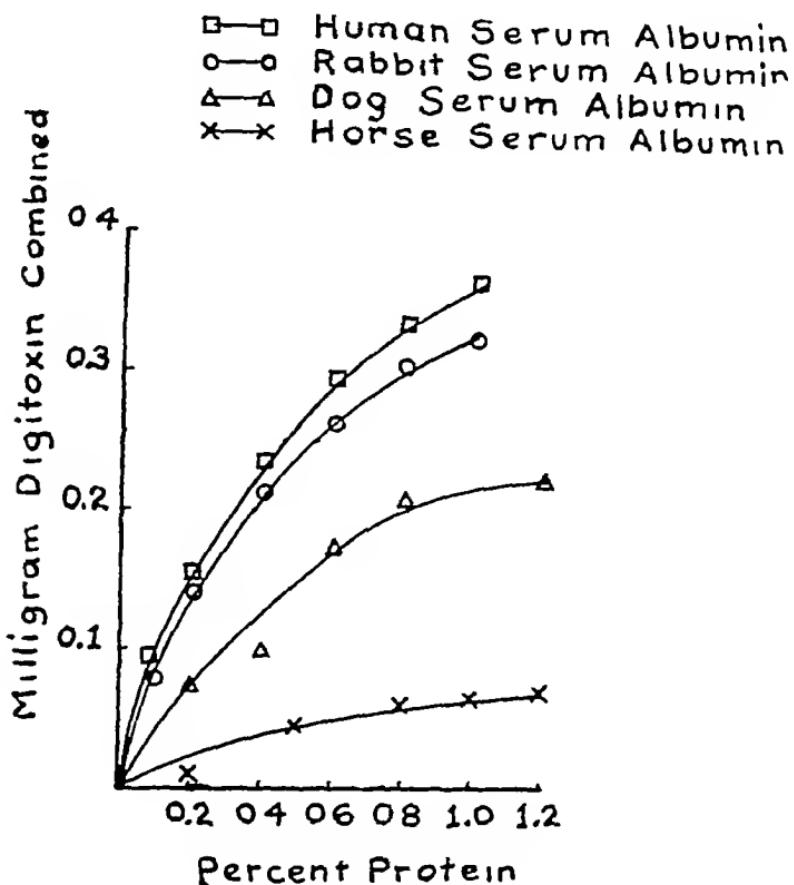


FIG 1 THE COMBINING ABILITY OF SERUM ALBUMINS OF DIFFERENT SPECIES WITH DIGITOXIN HOFFMANN LA ROCHE

Digitoxin concentration kept constant at 1:30,000. Albumin concentration varied as indicated.

protein was increased. A similar phenomenon appears when our data in fig 1 is expressed in a similar manner.

II The results of the experiment which was designed for the comparison of the combining power and protective action of rabbit serum with a number of cardio-active glycosides, are presented in table 3. The glycosides employed were fohandrin, digitoxin, digilanid and g- and k-strophanthin. For purposes of comparison of protective action it was essential to use concentrations of these

glycocones which would give equivalent effects on the isolated frog heart. The concentrations (which were found to give equivalent effects on the frog heart) were as follows: foliandrin 1 300,000, digitoxin 1 100,000, digilanid 1 100,000, *k*-strophantin 1 400,000 and *g*-strophantin 1 150,000. It can be seen from table 3 that the neutralizing effect of rabbit serum was most marked

O—O Rabbit Serum Albumin 0.5 per cent  
 X—X Horse Serum Albumin 0.5 per cent

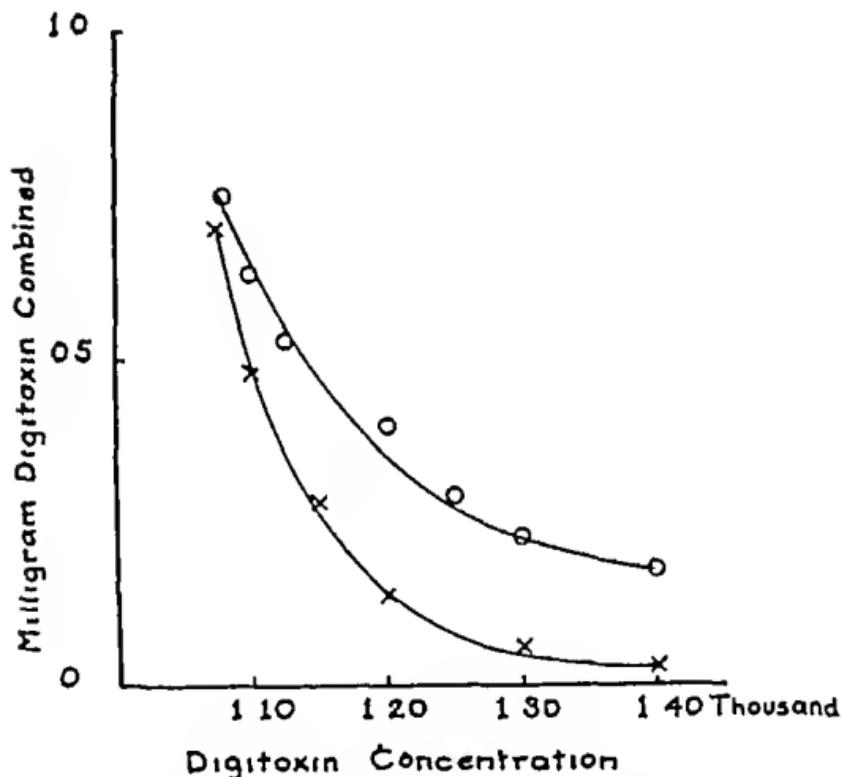


FIG. 2 THE COMBINING ABILITY OF SERUM ALBUMINS OF DIFFERENT SPECIES WITH DIGITOXIN HOFFMANN LA ROCHE

Protein concentration kept constant at 0.5 per cent protein. Digitoxin concentration varied as indicated

with foliandrin, less with digitoxin and least with digilanid. *g* and *k*-strophantin in the concentrations employed were not inactivated by rabbit serum or serum albumin. Reference to the last column of table 3 which contains the data on the combining power of these glycocones with the same specimen of rabbit serum which was used for the protection tests, shows that the relative degree of combining power of these glycocones is parallel to the degree of protection.

were much less apparent. The combining power of rabbit and horse serum albumin were about equal when a 1:7,500 digitoxin concentration was used (fig 2). Goigner and Pauly (15), while studying the combination of silver and protein, noted that if combining power was calculated on the basis of per gram protein, there was a progressive reduction of combining power as the concentration of

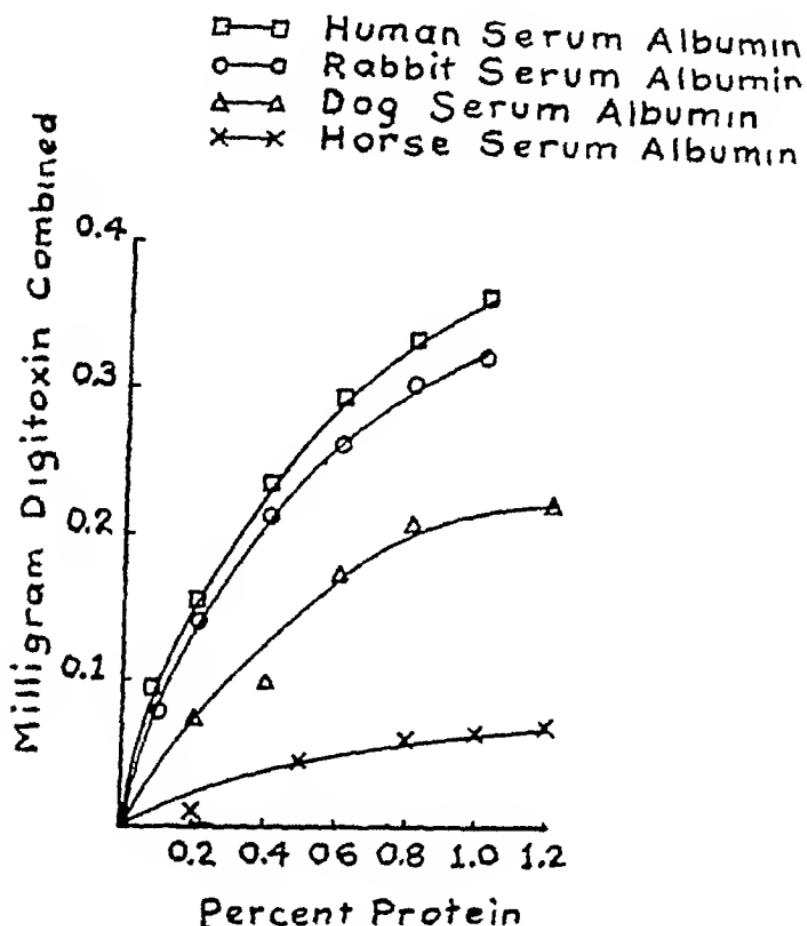


FIG. 1 THE COMBINING ABILITY OF SERUM ALBUMINS OF DIFFERENT SPECIES WITH DIGITOXIN HOFFMANN LA ROCHE

Digitoxin concentration kept constant at 1:30,000. Albumin concentration varied as indicated.

protein was increased. A similar phenomenon appears when our data in fig. 1 is expressed in a similar manner.

II The results of the experiment which was designed for the comparison of the combining power and protective action of rabbit serum with a number of cardio-active glycosides, are presented in table 3. The glycosides employed were sôhandrin, digitoxin, diglanid and g- and k-strophanthin. For purposes of comparison of protective action it was essential to use concentrations of these

TABLE 3—Continued

	NUMBER OF HEARTS USED	NUMBER OF HEARTS SHOWING EFFECT					TIME IN MINUTES FOR MAXIMUM EFFECT	NUMBER OF HEARTS SHOWING RECOVERY WITHIN 60 MIN.	COMBINING POWER OF SERUM AT 0.5 PER CENT PROTEIN AND 1:10,000 GLYCOSIDE CONCENTRATION MG. GLYCOSIDE PER GRAM PROTEIN
		+++	++	+	+	-			
Digilanid 1 50,000 in 1 per cent rabbit serum protein	6	0	0	0	2	4	24	0	
Digilanid 1 100,000 in 1 per cent rabbit serum protein	6	0	0	0	4	2	39	0	
Digilanid 1 150,000 in 1 per cent rabbit serum protein	8	0	0	2	6	8	29	1	1 181
Digilanid 1 225,000 in 1 per cent rabbit serum protein	6	0	3	3	0	0	36	3	
K-strophanthin 1 200,000 in Locke's solution	6	0	0	0	0	6	11	0	
K-strophanthin 1 400,000 in Locke's solution	6	0	0	0	0	6	24	0	
K-strophanthin 1 200,000 in 1 per cent rabbit serum protein	6	0	0	0	0	5	12	0	None
K-strophanthin 1 400,000 in 1 per cent rabbit serum protein	6	0	0	0	1	5	22	0	
g-strophanthin 1 150,000 in Locke's solution	6	0	0	0	4	2	33	0	
g-strophanthin 1 300,000 in Locke's solution	6	0	1	0	5	0	44	0	
g-strophanthin 1 150,000 in 1 per cent rabbit serum protein	6	0	0	0	6	0	23	0	None
g-strophanthin 1 300,000 in 1 per cent rabbit serum protein	6	0	0	1	5	0	48	0	
K-strophanthin 1 400,000 in 1 per cent rabbit serum albumin	8	0	0	0	1	7	25	0	None
g-strophanthin 1 300,000 in 1 per cent rabbit serum albumin	8	0	0	2	5	1	44	0	None

g and k-strophanthin, which were not neutralized by rabbit serum also failed to combine with the serum protein of either rabbit, dog or horse in any of the several concentrations used at hydrogen ion concentrations varying between pH 4.5 to 8.6. As with digitoxin, folicin and digilanid combined only with the albumin fraction and not with the globulin of rabbit serum.

As previously reported for digitoxin the denaturation by boiling or by alcohol

of serum protein which has combined with foliandrin and diglanid results in a liberation of these glycosides

III The aglucones, foliandrogenin, digitoxigenin and strophanthidin, were tested by means of the protection technic only, since the limited quantities available did not permit the performance of *in vitro* combination experiments. However, it is apparent from table 4 that foliandrogenin was inactivated by rabbit serum more than digitoxigenin, while strophanthidin was not inactivated at all. From our limited data it can be inferred that combination of the aglucones runs parallel to the combination of the respective glycosides. Furthermore, we con-

TABLE 4

*The ability of rabbit serum to protect against a number of cardiac aglucones*

	NUMBER OF HEARTS TESTED	NUMBER OF HEARTS SHOWING EFFECT					TIME FOR MAXIMAL EFFECT IN MINUTES	NUMBER OF HEARTS SHOWING RECOVERY WITHIN 30 MINUTES
		+++	++	+	+-	-		
Foliandrogenin 1 50,000 in Locke's solution	6	0	0	0	2	4	14	0
Foliandrogenin 1 100,000 in Locke's solution	6	0	1	2	2	1	13	1
Foliandrogenin 1 50,000 in 1 per cent rabbit serum protein	6	2	4	0	0	0	21	3
Foliandrogenin 1 100,000 in 1 per cent rabbit serum protein	6	6	0	0	0	0		6
Digitoxigenin 1 50,000 in Locke's solution	6	0	0	0	3	3	13	0
Digitoxigenin 1 100,000 in Locke's solution	6	0	0	1	2	3	17	0
Digitoxigenin 1 50,000 in 1 per cent rabbit serum protein	6	0	0	3	3	0	19	1
Digitoxigenin 1 100,000 in 1 per cent rabbit serum protein	6	1	5	0	0	0	22	3
Strophanthidin 1 100,000 in Locke's solution	6	0	0	0	2	4	15	0
Strophanthidin 1 100,000 in 1 per cent rabbit serum protein	6	0	0	0	1	5	17	0

clude that the sugar radicals of foliandrin and digitoxin do not play a predominant rôle in the combination of these glycosides with rabbit serum protein.

IV Certain factors affecting the combination of digitoxin with serum albumin have been studied in an attempt to get some insight into the mode of combination. Figures 1 and 2 show that the concentration of both protein and digitoxin are important in determining the degree of combination. Maximum combination could not be reached because digitoxin precipitated when the concentration in the dialysate exceeded 1 25,000. The maximum degree of combination obtained was 12.66 mg per gram of protein. This was determined for a rabbit serum albumin specimen at 0.5 per cent protein and 1 5,000 digitoxin concentration.

The combination of digitoxin with rabbit serum protein was not modified by the following procedures: changes of hydrogen ion concentration between pH 4.2 and 9.0, the doubling of the concentration of calcium and potassium ions in the Locke solution, the reduction of surface tension by the addition of saponin or bile salts in concentrations of 1:10,000 and dialysis of the serum for forty-eight hours prior to determining combining power. The denaturation of rabbit serum protein by heat or by 2:1 ethyl alcohol prior to its use abolishes completely its ability to combine with digitoxin.

V. The following experiments were performed to determine whether the combination of digitoxin with rabbit serum protein would modify the lethal dose of this glycoside for the intact anesthetized cat and dog. From table 5 it can be seen that in neither of these animals was there any appreciable modification of the L.D. of digitoxin when infused as a solution in 5 per cent rabbit serum protein.

TABLE 5

*The lethal dose for cats and dogs of digitoxin Hoffmann La Roche dissolved in saline or in 5% rabbit serum protein*

1:50,000 digitoxin solution was infused into the left femoral vein and blood pressure recorded from the right carotid artery. As anesthesia 0.04 gm. Nembutal Abbott was given intraperitoneally. All animals received artificial respiration with a Palmer respiration pump.

CATS		DOGS	
L.D. of digitoxin in saline mg. per kg. body weight	L.D. of digitoxin in 5% rabbit serum protein mg. per kg. body weight	L.D. of digitoxin in saline mg. per kg. body weight	L.D. of digitoxin in 5% rabbit serum protein mg. per kg. body weight
0.423	0.543	0.716	0.588
0.402	0.510	0.733	0.794
0.432	0.488	0.560	0.585
0.541	0.653	0.776	0.760
0.506	0.411	0.818	0.820
0.562	0.309	0.854	0.490
Average 0.477	0.488	0.742	0.679

Since rabbit serum failed to protect in the intact animal it occurred to us that this failure might be due to the ability of some organ to split the digitoxin protein complex infused. Therefore, experiments were carried out on the H.L.P. of the dog as a procedure more strictly analogous to the protection tests carried out on the isolated heart of the frog. The H.L.P. was infused, at a slow constant rate with a 1:50,000 solution of digitoxin dissolved either in saline or 5 per cent rabbit serum protein. Early experiments showed that the time required for inducing cardiac arrest was an important factor in determining the L.D. and must be established as a constant. The L.D. of 8.95 micrograms digitoxin per gram heart which was determined by us was obtained when the experimental period was 89-135 minutes. This is not the *minimal lethal dose* (M.L.D.) since the extension of the experimental period up to 220-240 minutes by means of a slow infusion rate of digitoxin would give a L.D. of only 6 micrograms of digitoxin per gram of

heart. Table 6 presents our experimental data, and it can be seen that the presence of serum had no influence on the L.D. of digitoxin for the dog H.L.P. under the experimental conditions described above.

Since we were not determining the M.L.D. of digitoxin for the dog H.L.P., it was possible that the *reaction time* might have been influenced by the presence

TABLE 6

*The lethal dose, for the dog heart lung preparation of digitoxin Hoffmann La Roche dissolved in saline or 5% rabbit serum protein*

H.L.P. Anesthesia ether, chloralose 90 mg./kg.

Blood Volume 800-850 cc

Blood Temperature 39.0-39.3°C

Resistance 70 mm. Hg.

Only hearts showing no irregularities and no decompensation are included. Slow continuous infusion of digitoxin in saline or 5% rabbit serum protein was made into the venous end of the circulation.

TIME IN MINUTES FROM START OF INFUSION UNTIL DEATH OF HEART	L.D. OF DIGITOXIN DISSOLVED IN SALINE GAMMA PER GRAM HEART	TIME IN MINUTES FROM START OF INFUSION UNTIL DEATH OF HEART	L.D. OF DIGITOXIN DISSOLVED IN 5 PER CENT RABBIT SERUM PROTEIN GAMMA PER GRAM HEART
116	8.35	134	7.88
98	9.25	109	8.40
112	8.83	98	9.63
100	9.48	106	9.04
Average 106.3	8.98	111.7	8.74

TABLE 7

*The reaction time of digitoxin Hoffmann La Roche in the H.L.P. of the dog with and without rabbit serum protein*

Blood Volume 800-850 cc

Temperature 39-39.2°C

Resistance 70 mm. Hg.

Digitoxin in 70 cc. saline or 5 per cent rabbit serum protein was added all at once into the venous reservoir. Figures are time in minutes from the addition of digitoxin until standstill of the heart occurred.

DIGITOXIN 1,500,000 IN SALINE	DIGITOXIN 1,500,000 IN 5% RABBIT SERUM PROTEIN	DIGITOXIN 1.5 MIL. IN SALINE	DIGITOXIN 1.5 MIL. IN 5% RABBIT SERUM PROTEIN
min	min	min	min
24	26	64	68
28	29	72	61
21			
Average 24.3	27.5	68.0	64.5

of the rabbit serum. We have studied the influence of rabbit serum on the reaction time of digitoxin with two different digitoxin concentrations, namely 1,500,000 and 1,1,500,000. To obtain these concentrations in the circulating blood the total dose of digitoxin dissolved in 70 c.c. of saline or five per cent rabbit serum protein was instantaneously added into the venous reservoir of the H.L.P. and

the lapse of time required to produce cardiac arrest noted. From table 7 it can be seen that the reaction time is not influenced by the presence of rabbit serum protein in concentrations of 0.5 per cent. This is in contrast to the fact that a similar concentration of serum protein has a definite effect on the isolated frog heart (2) and on the guinea pig heart perfused with Ringer solution (16).

**Discussion** From our results it is clear that at low digitoxin concentrations there are definite differences in the combining powers of serum proteins from various species. The combining powers determined at 0.5 per cent protein and 1/30,000 digitoxin concentration can be correlated with the protective properties of any given serum albumin specimen. On the other hand, when high digitoxin concentrations are employed to determine combining power, the species differences are much less apparent and there is no longer any correlation between combining power and protective action. The inability of Lendle et al. to demonstrate species differences in the combining powers of serum albumins may possibly be due to their use of high glycoaide concentrations for determining combining powers. Since the original papers of Lendle et al. are not available to us, we are in no position to make any critical analysis of their work or to offer any further explanations.

Bennhold (17) has offered the interesting hypothesis that serum protein combinations play an important rôle in the circulatory transport of various substances. We have tested this hypothesis by attempting to correlate our data on the combining power of serum proteins of different species with cardiac glycoaides and aglucones at low glycoaide concentration with certain known pharmacological reactions namely, lethal dose cumulation washability (reversibility in the isolated frog heart), reabsorbability from the gastro-intestinal tract, and water solubility of the cardio-active glycoaides. We were unable to establish any significant correlation of these phenomena and Bennhold's hypothesis does not apply to the phenomena under discussion.

The question arises whether the combination of serum proteins with cardio-active glycoaides is a so-called adsorption reaction or a stoichiometrical combination. We have applied the Freundlich formula to some of our data. When plotting  $X/M$  against  $\log C$  where  $X$  is the amount of adsorbed glycoaide  $M$  the amount of protein, and  $C$  the concentration of unadsorbed glycoaide we should get a straight line if the combination is of the adsorptive type. Since the resulting lines obtained by us were curves one must conclude that the combination of digitoxin to serum albumin is not purely an adsorption reaction. On calculating the quantities of digitoxin which combine with serum albumin, it is likewise not a pure stoichiometrical combination. One is impressed by the similarity of the types of curves obtained in the course of our experiments to those obtained in quantitative studies of antigen antibody combination (18). These data probably indicate the complexity of the factors involved rather than the mechanism of combination.

It is clear that the sugar molecule in the cardio-active glycoaides is not essential for their combination with serum proteins, since both the aglucones of folandrin and digitoxin combine readily with rabbit serum proteins. What portion of the

aglucone is responsible for combination cannot be decided on the basis of our data.

The experiments on intact cats and dogs and on the H L P of the dog show that under the conditions of these experiments the combination of digitoxin with rabbit serum protein does not influence the L D or the reaction time of this glycoside. Hoekstra (4) and Genuit and Eschbach (16) have shown that isolated rabbit and guinea pig hearts perfused with Tyrode's solution can be protected against digitoxin by a foreign serum, but not by the species specific serum. Whether the same holds true for the isolated dog heart perfused with Tyrode's solution is not clear, since in our experience rabbit albumin concentrations of 0.1 per cent have been so toxic to the heart that these experiments had to be discontinued. That the H L P of the dog is not protected against digitoxin by the presence of a foreign serum is an interesting phenomenon which is poorly understood. Whether the temperature of the perfusion fluid or the presence of species specific serum modify this protection are some of the factors still to be studied.

Based on his findings that the isolated frog heart is not protected against digitoxin by frog serum, Hoekstra has formulated the hypothesis that the digitoxin-serum protein complex penetrates the cardiac cell wall if digitoxin is combined to species specific serum, but does not do so in combination with a foreign serum. Brücke's (14) and our own experiments regarding the protective action of frog serum invalidate some of the considerations and conclusions of Hoekstra. The demonstration that frog serum protects only slightly is best explained on the basis that its combining power at low digitoxin concentrations is very low.

#### SUMMARY

The combination of digitoxin with sera and serum albumins of different species has been studied. At low digitoxin concentrations we find definite differences in the combining powers of serum proteins from various species. Using digitoxin concentrations above 1:10,000 these species differences become much less apparent. There is a positive correlation of combining power with the relative ability of various serum proteins to protect against the cardiotoxic effects of digitoxin only when digitoxin concentrations below 1:20,000 are employed.

A number of cardio-active glycosides and aglucones have been examined for ability to combine with rabbit serum. Foliandrin, digitoxin and digilanid combine with serum proteins, while both g- and k-strophanthidin do not. Whereas the aglucones of foliandrin and digitoxin combine with serum proteins, strophanthidin does not. A number of factors influencing digitoxin-albumin combination have been studied, and no conclusion could be reached regarding its nature.

Under our experimental condition, the digitoxin-rabbit serum protein combination does not influence either the L D or the reaction time of digitoxin in the intact anesthetized cat and dog, and the H L P of the dog.

I wish to express my thanks to Dr E. W. Dennis, Chairman of the Department of Bacteriology and Parasitology of the American University of Beirut, for his assistance in the preparation of the manuscript.

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## ABSORPTION OF SINGLE DOSES OF IRON<sup>1</sup>

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Recognition of iron as a necessity for hemoglobin synthesis has given rise to voluminous literature which has proved most valuable in the field of clinical medicine. Although most extensive studies are based on clinical or laboratory induced anemia, Moore et al (1-3), McCance and Widdowson (4-7), Leverton (8), and Johnstone (9), in recent publications report findings on normal individuals, human and animal. A review of this literature on normal individuals indicates that most of the work involved multiple doses and observation of the final endpoint. The studies reported herein were undertaken for the purpose of evaluating the relationship of dosage to the rapidity and degree of absorption in normal animals following a single dose of an iron compound, using the serum iron level as the basis for comparison.

Male and female rats weighing over 200 grams were used. These rats were of Wistar descent obtained from Sunnyhill Rat Farm. The iron compounds employed were Iron and Ammonium Citrate Merck U.S.P. Brown Powder containing Fe equivalent to not less than 16.5 and not more than 18.5 per cent, and Ferrous Sulfate Merck U.S.P. ( $FeSO_4 \cdot 7H_2O$ ) containing approximately 20.6 per cent Fe. This choice of iron compounds was made in order to study the ferric and ferrous forms. In Iron and Ammonium Citrate, however, the ferric iron is bound in an organic molecule of such nature that it does not react to the test for ferric salts U.S.P. XII. Each of these compounds was administered orally.

**METHOD OF PROCEDURE** The animals received by stomach tube doses of 0.1, 0.25, 0.5 and 1.0 gram of either iron salt dissolved in 5 cc of distilled water. At designated time intervals the rats were anesthetized by intraperitoneal administration of 3 per cent sodium pentobarbital solution and bled from the carotid artery, care being taken not to cause destruction of red cells. The time intervals were varied from 1 to 24 hours. The blood samples were centrifuged and the sera carefully separated. This sera was transparent, practically colorless, and slightly opalescent.

The method used for determining the serum iron levels was comparable to Kennedy's procedure (10).

1 Reagents used (a) C P Reagent Acid Sulfuric, (b) Reagent Acid Perchloric, 60 percent, (c) Reagent Acid Nitric Fuming, (d) Reagent Iso amyl Alcohol, and (e) Reagent Potassium Thiocyanate. All reagents were Merck Blue Label.

2 Digestion Either 1 or 2 cc of serum were pipetted into a 100 cc Kjeldahl flask. To this was added 2.5 or 5 cc respectively of reagent sulfuric acid. This mixture was digested in a hood over a moderate Bunsen flame until there was disintegration and complete solution of all solid particles. When cool 1 or 2 cc of perchloric acid were added and the mixture heated until all chlorine was driven off. The remaining colorless liquid was

<sup>1</sup> Acknowledgment is made of a grant from the Proprietary Association in support of this study.

then cooled. After adding 2 drops of fuming nitric acid the contents of the Kjeldahl flask were transferred to a 50 cc. volumetric flask and made to volume with distilled water at room temperature. Ten cc. of this mixture were pipetted into a cylindrical glass-stoppered 25 cc. graduate. Then 10 cc. of isoamyl alcohol and 5 cc. of a 20 percent solution of potassium thiocyanate were added. After shaking vigorously the solution was allowed to stand for a few seconds until complete separation of layers occurred.

3. Colorimetric comparison After setting the zero with iso-amyl alcohol 5 cc. of the colored iso-amyl alcoholic layer were poured or pipetted into a Klett tube and read by the Klett-Summerson Photoelectric Colorimeter using the green filter #54. Reagent blanks were run concurrently and readings adjusted.

4. Iron Standard Solution One hundred milligrams of Reagent Iron Wire (Merck Blue Label) were washed with ether to remove all dirt and grease weighed exactly and placed in a liter flask to which 20 cc. of 10 per cent sulfuric acid were added. This was shaken frequently and allowed to stand until the iron wire was in solution which took four days. Then 6 cc. of concentrated nitric acid were added and the solution made to volume with distilled water. This dilution of 0.1 mg. per cc. required dilution to 0.005 mg. per cc. so that readings on 2 cc. would fall within the range of those on 2 cc. of serum.

5. Calculation Colorimeter readings were converted to micrograms per cent of serum iron according to the following formula:

$$\frac{500}{S} \times R = \text{micrograms per cent}$$

500 = micrograms of Fe per 100 cc. of standard

S = reading of standard (mean)

R = reading of serum unknown.

Whenever possible 2 cc. of serum were used but occasional determinations were based upon 1 cc. in which case the reading was multiplied by 2.

**EXPERIMENTAL DESIGN** Controls consisted of animals sacrificed without any dosage or animals administered 5 cc. distilled water before bleeding for sera. The first compound studied was Iron and Ammonium Citrate. For the most intensive study male rats were treated with this compound using 0.1, 0.25, 0.5 and 1.0 gram per rat, varying time intervals from  $\frac{1}{2}$  to 24 hours. A smaller number of females were treated in the same manner. To approximate common usage of iron preparations the animals were allowed food and water at all times. Upon tabulation of data and plotting of graphs it was apparent that maximum absorption of these doses had been obtained. The 1.0 gram dose proved toxic while the 0.1 gram dose gave readings of little or no significance. These two doses therefore, established the limits of dosage. All data were collected and tabulated using the Fisher Table (11) of  $t$  to evaluate the significance of results. For purposes of this investigation a 'P' value of 0.05 or less is considered significant.

With this picture completed Ferrous Sulfate was studied using a representative number of animals. Time intervals were varied from  $\frac{1}{2}$  to 24 hours with stress placed upon those intervals of most significance as shown by the previous work on Iron and Ammonium Citrate. These data were also tabulated and statistically evaluated.

A small number of animals were fatigued in a mechanically driven tin cylinder which did not allow eating or resting periods. After six hours 0.5 grams of Iron and Ammonium Citrate was administered and the animals sacrificed  $\frac{1}{2}$  hour later. The same procedure was followed on a few animals fasted for six

hours, but not fatigued. Controls were fasted for the same period or fatigued while fasting. Likewise, a small number of animals were fasted from 16-18 hours, then given the range of doses previously used, again selecting the most significant time intervals. A control for this phase of the problem was fasted for the same length of time.

In a separate series of experiments, each dose of Iron and Ammonium Citrate was administered hourly until death occurred or until a total of six doses had been received.

It is important to note that these studies on the absorption of a single dose of iron present four variable factors—the iron compound used, the dosage, the sex and the time interval. The results obtained are summarized in tables 1 and 2.

**DISCUSSION OF RESULTS** Observation of table 1, Group A, shows that the dose of 0.1 gram of Iron and Ammonium Citrate, although slightly slow in its action to increase the serum iron level, gives significant results within the 4 to 6 hour interval with a percentage increase comparable to that of the next larger dose, 0.25 gram. However, the 0.25 gram dose of table 1 (B) has a longer period of significant values, ranging from the  $\frac{1}{2}$  to the 4 hour interval with the maximum absorption at three hours. In contrast to table 1 (A) and 1 (B) the larger doses of table 1 (C) and 1 (D) show significant results as early as  $\frac{1}{2}$  hour and as late as the end of the sixth hour. The peak in both these groups is three hours, the same as in table 1 (B). In table 1 (C) the reading for the 45 minute interval surpasses all other readings, but when compared statistically with the three hour peak it is not significantly different. Both table 1 (C) and 1 (D) show an identical percentage increase over controls. The 1.0 gram dose proved very toxic throughout and diarrhea was marked within the first hour. A few individuals of the longer time intervals died from this toxic dose. This condition did not exist following the use of the smaller doses. Table 1, Groups A, B, C and D show no significant values after the six hour interval.

The response to Iron and Ammonium Citrate by females presents a somewhat different picture. Since table 1 (E) shows no significant increase in serum iron, 0.1 gram delineates the lower level of effective dosage. The 0.25 gram dose in table 1 (F) is significant only at the 2 hour interval. Table 1 Groups G and H show the effectiveness of 0.5 and 1.0 gram doses in female rats. Both these doses show a range of significant results more comparable to the males. The 0.5 gram dose has significant readings extending from the  $\frac{1}{2}$  hour interval through nine hours, except for the six hour reading. The maximum absorption was obtained in four hours. The 1.0 gram dose shows significant results for absorption within the first  $\frac{1}{2}$  hour but lasting only through the third hour. This is in general agreement with Moore et al (1) who found large doses of Iron and Ammonium Citrate gave an apparent rise in serum iron levels within the first half hour but that the maximum absorption ranged from two and one half to five hours, and with Barer and Fowler (12) who reported more rapid but not more pronounced effects with larger doses of Iron and Ammonium Citrate. Female rats receiving the 1.0 gram dose showed distress and more marked diarrhea than male rats. This perhaps accounted for the short duration of

TABLE 1

The absorption of single doses of iron compounds at varying intervals after oral administration using the serum iron level as the basis for evaluation

The significance of the results (P) is estimated by the Fisher t method

GROUP AND Dose	Dose	SEX	No. of Rats	TIME INTERVAL	SERUM IRON			
					micrograms per cent	micrograms per cent	P	Increase over control
	gm./rat			hours				
Control group		male	14		303	93-460		
		male	3	1	411	403-512	0.1	
	0.1	male	3	2	404	323-447	0.3	
	0.1	male	3	3	303	295-500	0.3	
	0.1	male	4	4	484	333-605	0.05	
	0.1	male	3	5	513+	402-654	0.05*	60.3
Group A Iron and Ammonium Citrate	0.1	male	1	5	400		0.0**	
	0.25	male	8	1	347	144-633	0.5	
	0.5	male	5	1	422	165-939	0.3	
	0.25	male	6	1	410	337-500	0.05*	
	0.25	male	9	1	463	303-637	0.01*	
	0.25	male	0	2	488	139-760	0.01	
	0.5	male	8	3	633+	423-1096	<0.01	
	0.25	male	5	4	510	309-926	<0.01	
	0.25	male	5	5	393	257-500	0.2	
	0.25	male	4	5	381	181-468	0.5	
	0.25	male	2	9	304	254-321	0.9	
	0.25	male	3	12	163	123-202	0.3	
	0.25	male	2	24	203	203-287	0.8	
Group C Iron and Ammonium Citrate	0.5	male	14	1	535	361-910	<0.01	
	0.5	male	18	1	603	433-977	<0.01*	
	0.5	male	5	1	887	500-1827	<0.01*	
	0.5	male	14	1	731	232-1256	<0.01*	
	0.5	male	8	2	658	349-1256	<0.01*	
	0.5	male	8	3	771+	521-1105	<0.01*	
	0.5	male	5	4	689	380-1112	<0.01*	
	0.5	male	4	5	738	568-835	<0.01	
	0.5	male	3	5	622	537-677	<0.01	
	0.5	male	2	9	379	288-499	0.6	
	0.5	male	2	12	318	214-421	0.3	
	0.5	male	5	24	228	116-361	0.6	
Group D Iron and Ammonium Citrate	1.0	male	5	1	709	400-1000	<0.01*	
	1.0	male	11	1	659	163-1386	<0.01*	
	1.0	male	10	1	728	537-1147	<0.01*	
	1.0	male	1	1	673	423-1140	<0.01*	
	1.0	male	7	2	702	419-1001	<0.01*	
	1.0	male	5	3	833+	720-1235	<0.01*	
	1.0	male	5	4	583	365-789	<0.01*	
	1.0	male	5	5	646	121-1335	<0.01	
	1.0	male	2	6	603	429-707	0.05	
	1.0	male	2	9	303	235-551	0.5	
	1.0	male	1	12	425		0.2	
	1.0	male	3	24	106	109-344	0.3	

TABLE 1—Continued

GROUP AND DRUG	DOSE	SEX	NO OF RATS	TIME INTERVAL	SERUM IRON			
					Mean	Range	P	Increase over control†
	gm/rat			hours	micrograms per cent	micrograms per cent		
Control group		female	7		394	279-558		
Group E Iron and Ammonium Citrate	0.1	female	2	1	427	402-451	0.8	
	0.1	female	2	2	418	316-519	0.9	
	0.1	female	2	3	484	416-551	0.5	0.0
	0.1	female	2	4	575	395-754	0.3	
	0.1	female	1	5	412		0.7	
	0.1	female	2	6	426	409-442	0.8	
Group F Iron and Ammonium Citrate	0.25	female	4	1	721	437-1349	0.1	
	0.25	female	3	2	572+	544-621	0.05*	45.1
	0.25	female	3	3	531	500-588	0.1	
	0.25	female	3	4	625	575-666	0.3	
	0.25	female	3	5	323	233-502	0.5	
	0.25	female	3	6	336	35-699	0.7	
	0.25	female	1	9	451		0.5	
	0.25	female	1	12	149		0.2	
	0.25	female	1	24	377		0.3	
Group G Iron and Ammonium Citrate	0.5	female	2	½	687	582-791	0.5	
	0.5	female	6	½	685	363-1028	0.02*	
	0.5	female	10	1	833	380-1782	0.02*	
	0.5	female	5	2	810	233-1175	0.02*	
	0.5	female	3	3	792	775-809	<0.01*	94.5
	0.5	female	3	4	936+	395-1335	0.05*	
	0.5	female	3	5	688	447-812	0.05*	
	0.5	female	2	6	337	270-395	0.6	
	0.5	female	1	9	535		0.02*	
	0.5	female	1	12	230		0.01	
	0.5	female	1	24	360		0.5	
Group H Iron and Ammonium Citrate	1.0	female	2	½	1019+	698-1340	0.02*	
	1.0	female	2	½	977	930-1023	<0.01*	
	1.0	female	6	1	931	233-2038	0.05*	
	1.0	female	3	2	703	368-944	0.05*	
	1.0	female	3	3	771	612-975	0.01*	
	1.0	female	3	4	593	433-882	0.2	
	1.0	female	3	5	578	461-688	0.1	
	1.0	female	3	6	520	337-807	0.3	
	1.0	female	1	12	386		0.9	
	1.0	female	1	24	128		0.01	
Group I Ferrous Sulfate	0.1	male	1	1	423		<0.01*	
	0.1	male	1	2	760+		<0.01*	86.0
	0.1	male	1	3	512		<0.01*	

TABLE I—Continued

GROUP AND DRUG	DOSE	SEX	NO. OF RATS	TIME INTERVAL	SERUM IRON			
					Hours	micrograms per cent	Range	P
	gm./rat					micrograms per cent		per cent
Group J Ferrous Sulfate	0.25	male	3	1	732+	564-1023	<0.01*	
	0.25	male	3	2	681	642-718	<0.01	
	0.25	male	3	3	707	569-891	<0.01	
	0.25	male	4	4	635	433-884	<0.01	
	0.25	male	1	24	605		<0.01	
Group K Ferrous Sulfate	0.5	male	2	1	1018+	481-1564	0.01*	
	0.5	male	1	1	963		<0.01	
	0.5	male	3	1	839	605-1005	<0.01*	
	0.5	male	3	2	688	181-1180	0.02*	
	0.5	male	3	3	762	384-1001	<0.01*	
	0.5	male	2	4	633	395-872	0.05*	
	0.5	male	1	6	343		0.2	
Group L Ferrous Sulfate	1.0	male	2	1	1068	977-1153	<0.01	
	1.0	male	3	2	845	568-1209	<0.01*	
	1.0	male	2	3	811	442-779	0.05	
	1.0	male	1	4	1642+		<0.01	
	1.0	male	1	6	102		<0.01	
Group M Ferrous Sulfate	0.1	female	1	1	989+		<0.01	
	0.1	female	1	2	826		<0.01	
	0.1	female	1	3	802		<0.01	

\* Maximum or peak absorption.

† Significant increases in serum iron level.

† This value is based on the mean of the significant increases only.

significant results as compared to table I (D) on males. Death occurred in a small number of animals after the first hour and autopsy showed extreme irritation of the intestinal tract. For equal doses the percentage increase was less in females than in males.

In contrast to the results discussed on Iron and Ammonium Citrate, results from each of the doses of Ferrous Sulfate are highly significant. In general the maximum levels are reached earlier with the sulfate than with the citrate. The percentage increases are considerably higher in all groups treated with Ferrous Sulfate than in those treated with Iron and Ammonium Citrate. These results are summarized in Table I Groups I, J, K, L and M. The apparent advantage of increased rapidity and degree of action is overshadowed by the fact that

toxicity was observed in all groups except those administered the smallest dose. For purposes of comparison it should be noted that the degree of absorption after 0.25 gm of Ferrous Sulfate is approximately equal to the absorption after 0.5 gm of Iron and Ammonium Citrate. Throughout these studies the ferric compound has been less irritating than the ferrous compound. Since, however, the ferric citrate is not highly ionized, this observation is not a generalization which would include the inorganic ferric salts. From the small number of experiments summarized in table 1, Group M, it would appear that the sulfate effectiveness is greater in females than in males.

The results obtained from fasted and fatigued animals are summarized in table 2, Groups A, B, C and D. The serum iron level for each control was comparable and the readings were therefore combined. Treated individuals of Groups A and B show comparable results for fasting without fatigue and fasting while being fatigued. In view of these results a representative number of animals were fasted without fatiguing for 16 to 18 hours, then administered doses of 0.25, 0.5 and 1.0 gram of Iron and Ammonium Citrate and sacrificed at two or three hour intervals to coincide with the highly significant peaks of absorption as summarized in table 1.

Some investigators feel that fasting is necessary for uniform results. If, however, Groups A, B, C and D of table 2 are compared with appropriate groups of table 1, it is apparent that the maximum degree of absorption is comparable under each circumstance and that fasting or fasting with fatiguing does not appreciably increase the absolute maximum level. With the dose of 0.25 gram of Iron and Ammonium Citrate the maximum absorption appears earlier in the fasted animals, but is not significantly greater. Similarly, the percentage increase over normal controls is comparable.

On the other hand the per cent of increase in fasted animals over the fasted controls is markedly higher. Therefore, it appears obvious that the only fundamental difference in the two groups is the lower serum iron control value in the fasted animals.

To determine whether the serum iron level obtained from a single dose of Iron and Ammonium Citrate represents the maximum absorption attainable within a short period of time, a series of hourly doses were administered. All animals succumbed between the third and sixth hour except two individuals. One male rat surviving six hourly doses of 0.1 gram gave a reading of 593 micrograms, a 15 per cent increase over the peak of absorption shown in table 1 (A) where a single 0.1 gram dose was used. In comparing the same individual with Table 1 (C) where a single dose of 0.5 gram was given, the reading falls between those of  $\frac{1}{2}$  and  $\frac{1}{3}$  hour but far below the remaining significant results. The individual receiving six hourly 0.25 gram doses gave a reading of 547 micrograms against the peak for a single dose of 633 as shown in table 1 (B). Remembering that this particular individual received 1.5 grams over the six hour period, the serum iron level did not compare with any group receiving a single dose of 1.0 gram as shown in table 1 (D).

TABLE 2

The absorption of single doses of Iron and Ammonium Citrate orally administered after periods of fasting or fasting and fatiguing

The significance of the results (P) is estimated by the Fisher *t* method

GROUP	DOSE	SEX	NO. OF RATS	TIME INTERVAL	SERUM IRON			
					Mean	Range	P	INCREASE OVER CONTROL
	gm./rat			hours	micrograms per cent	micrograms per cent		per cent
Control group		male	14		303	93-485		
Control group		female	7		394	279-558		
Control group Fasted and fatigued		male	3		188	123-167		
Group A Fasted and fatigued for 6 hours	0.5	male	4	1	602	507-958	0.01*	128 401 F
Group B Fasted for 6 hours	0.5	male	1	1	507		<0.01	67 267 F
Group C Fasted for 16-18 hours	0.25	male	1	2	515		<0.01*	141
	0.25	male	1	3	847		<0.01	429 F
	0.5	male	1	2	721		<0.01*	150
	0.5	male	1	3	705		<0.01*	594 F
	1.0	male	1	2	721		<0.01*	151
	1.0	male	1	3	802		<0.01*	451 F
Group D Fasted for 16-18 hours	0.25	female	1	2	1014		<0.01*	108
	0.25	female	1	3	632		<0.01	496 F
	0.5	female	1	2	893		<0.01	85
	0.5	female	1	3	868		<0.01	428 F
	1.0	female	1	2	1051		<0.01*	166 661 F

Significant increases in serum iron level. When compared to fasted and fatigued controls all P values are 0.01 or less.

\* This value is based on the mean of the significant increases only. 'F' indicates percentage increase over fasted and fatigued controls.

#### CONCLUSIONS

1 Iron and Ammonium Citrate when given to rats in effective doses was much less irritating to the intestinal tract than Ferrous Sulfate.

- 2 An effective dose of either iron compound need not be an irritating dose
- 3 Absorption of iron into the blood serum was almost immediate, but significant absorption took place up to six hours after administration
- 4 Evidence is presented to show that equally satisfactory results may be obtained whether the animals are permitted food or are fasted or fasted and fatigued Under any of these conditions the maximum serum iron level is comparable

5 Absorption of equal doses of Iron and Ammonium Citrate was greater in males than in females, whereas females showed the greater percentage increase following Ferrous Sulfate

6 The absorption following a single dose of iron compound was as great as that following repeated administration

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# ANTAGONISTS FOR THE CIRCULATORY DEPRESSION OF QUININE INJECTED INTRAVENOUSLY AND THE IMPLIED CHOLINERGIC ACTION AND NATURE AND IMPORTANCE OF THE VASODILA- TATION IN THE DEPRESSION

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Although quinine is ordinarily not injected intravenously in treating malaria partly because of its undesirable circulatory depression this method has been widely used in civilian practice and in the armed services for the embolic or cerebral form of the disease, or in coma. It was reported to have saved lives in the early South Pacific operations provided the injection was given early. This method might be expected to deliver promptly and effectively the quinine to the organs and capillaries where the parasites are stagnated, as in *P. falciparum* infection. However, malarial or battle casualties or both may be poor circulatory risks which might increase the hazard of the intravenous injection. Therefore it appeared desirable to us to obtain a better understanding of the actions of quinine intravenously and to discover if possible measures for preventing or mitigating the circulatory depression. This report presents the results of such a study.

A detailed discussion of previous recommendations (1 2 3 4 5 6, 7 and 8) for using quinine intravenously is omitted here to save space. Briefly however the common features are quinine dihydrochloride or hydrochloride in low concentration and slow injection. Exceptional concentrations of quinine are 5 per cent (8) and 10 per cent (7) which seem dangerous. Epinephrine 1 cc 1:1000 intravenously is advised as needed (6); the same dose is also given in mixture with the quinine (8) and the value of this has been confirmed experimentally by us.

**Methods** The general plan was to test the effects of antagonists on the quinine depression according to known effects of these agents. The aim was to prevent, correct or relieve the depression by mediating opposite actions on the known physiological elements involved and also to strike at the fundamental cause of quinine depression. A fall in blood pressure and slowing of the heart were the criteria of circulatory depression using rabbits and cats chiefly rabbits (76 rabbits and 14 cats total 90 animals). Rabbits were well suited to our purpose because of their labile circulation which presented variable states corresponding in general to good or poor circulatory risks. Cats were used chiefly for checks of suggestive anticolicope measures in rabbits and for the cholinergic mechanism and peripheral vasodilatation. Pentobarbital 30 mgm per kgm injected intraperitoneally was used for anesthesia in cats while urethane 0.75 gram per kgm rectally was used in rabbits.

Blood pressure was recorded from a mercury manometer joined usually to the right carotid artery using 20 per cent sodium thiosulfate as anticoagulant. Heart rate was counted from the oscillations of the manometer. Tests of the antagonists were made in animals with high or normal blood pressure and in those with low pressure produced by hemorrhage. A standard single therapeutic dose of quinine sulfate was injected into a saphenous vein at a constant and moderately rapid rate from an ordinary graduated burette,

1 cc, 10 mgm per kgm of body weight in 2 minutes. This dose corresponded to 0.7 gm in a 70 kilo adult man. Occasionally, 30 mgm per kilo was given to cause profound depression. The total number of injections was about 1140 and of agents tested, 32. The antagonistic agents used were injected mixed with, or given 1 or 2 minutes before the quinine. Successive injections were usually made until death. Generally, time was allowed for recovery to the previous level before the next injection, although there was in all cases a progressive reduction in blood pressure. The rate of injection used was more rapid than that usually advised clinically, for malarial patients, but this was done purposefully so as to produce a prompt and sharp, though moderate, lowering of blood pressure, which would facilitate comparisons of the results. There would have been no object in making the injections so slowly as to avoid evident depression since this would have deprived us of a valuable criterion. However a few injections were made more slowly, approximating certain clinical recommendations, but without essential differences in the degree of depressor action.

Quinine sulfate was used by us, partly because of the availability of a supply of this salt, and partly, because it did not precipitate with human plasma and horse serum, while the quinine hydrochloride did in 1 per cent strength. The quinine sulfate was dissolved in 0.9 per cent sodium chloride solution, 1 cc containing 1.25 mgm quinine sulfate, so that 8 cc per kilogram of rabbit were injected for 10 mgm of the alkaloid. Injections of 8 cc of saline solution at the same rate as the quinine solution caused no, or only momentary, changes in blood pressure. From time to time the same dose of quinine base as quinine bisulfate in a total of 1 or 2 cc of saline solution was injected with exactly the same fall in blood pressure. Thus, the volume effect of the solution of quinine sulfate was negligible as a contributory factor to the depression in successive injections.

Variations from the general procedure were introduced according to the requirements of certain tests, and these are considered in the text. The results of all experiments were summarized according to per cent changes in blood pressure and heart rate after each agent or procedure and compared with the preceding or control level. The changes in blood pressure were found to be more consistent than those in pulse rate, despite the fact that the heart was invariably slowed by the quinine. The changes are expressed as averages of at least three, and generally more, injections of the more promising agents. At least 3 animals were used with important agents, with others, which appeared repetitious or less important, trials were limited to one animal. A statistical analysis of the results was not made, since the data for the different agents varied greatly, and was not deemed necessary, since the trends were sufficiently evident.

**CONTROLS** Injections of the standard dose of 10 mgm per kgm, of quinine sulfate in physiological salt solution in 55 rabbits with normal or high blood pressure produced an average fall of 29 per cent with complete recovery to the original level in 56 per cent of 206 trials (table 1). Injection of the same dose in 5 rabbits with blood pressures below 60 mm Hg produced a reduction of 30 per cent with complete recovery in 77 per cent of 13 trials (table 2). Doses of 30 mgm per kgm, in 10 rabbits with normal pressures, caused an average reduction of 39 per cent, with complete recovery in 64 per cent of 14 trials (table 1). When the same dose was injected in 4 rabbits with pressures below 60 mm Hg, there was a reduction of 52 per cent with complete recovery in 71 per cent of 7 injections (table 2). Definitely, the depressor action increased with the increase in dosage of quinine in both normal and low circulatory states. While the number of animals for the 2 different doses in the 2 states differed considerably, the average degree of the depression after the small doses was practically the same in the two circulatory states, but not quite comparable after the larger

TABLE 1

Effects of various agents with or prior to quinine sulfate injected intravenously in rabbits with normal blood pressure

AGENT	DOSE PER KGM.	NUMBER OF ANIMALS	NUMBER OF TRIALS	AVERAGE PER CENT FALL OF BLOOD PRESSURE AND RANGE	COMPLETE RECOVERY PER CENT OF TRIALS
Controls					
Saline solution		55	206	29 (4-83)‡	56
Saline solution		10	14	39 (15-56)	64
Colloidal agents					
Human plasma	8 cc	3	8	28 (12-45)	75
Human plasma	24 cc	2	8	43 (10-74)	67
Methocel 0.075%	8 cc	1	8	35 (22-49)	33
Acacia 6%	8 cc	3	6	24 (20-36)	100
Acacia 6%	24 cc	3	5	26 (10-35)	19
Acacia 16%	8 cc	2	4	18 (10-27)	75
Pectin 1%	8 cc	2	8	24 (7-36)	100
Pectin 3%	8 cc	3	7	42 (4-73)	86
Pectin 8%	24 cc	1	2	30 (30-31)	100
Pectin 6%	8 cc	1	3	14 (5-20)	100
Congo red†	50 mgm	4	4	26 (11-39)	80
Congo red †	50 mgm	4	4	63 (45-90)	50
Congo red†	100 mgm	3	3	42 (7-68)	86
Sympathomimetic amines					
Epinephrine	0.01 mgm	11	33	21 (0-64)	91
Epinephrine	0.015 mgm	4	10	13 (0-30)	100
Epinephrine	0.02 mgm.	9	31	17 (0-74)	97
Neosynephrine	0.1 mgm.	2	5	18 (11-31)	100
Neosynephrine	0.05 mgm	2	7	12 (3-18)	86
Benzedrine	5-15 mgm	1	2	57 (46-68)	100
Ephedrine	5.0 mgm.	2	5	27 (11-59)	100
Ephedrine	10.0 mgm	2	4	22 (8-31)	100
Ephedrine	15.0 mgm	3	4	24 (0-67)	100
Calcium salts					
Calcium chloride	10 mgm	6	27	16 (0-34)	100
Calcium chloride	20 mgm.	6	22	24 (0-65)	95
Calcium chloride	40 mgm	3	14	21 (8-63)	71
Calcium chloride	80 mgm.	4	6	44 (7-100)	50
Calcium lactate	26 mgm	3	9	17 (7-24)	67
CaCl <sub>2</sub> plus epinephrine	10 + .01 mgm	1	3	19 (15-21)	100
CaCl <sub>2</sub> plus epinephrine	20 + .01 mgm	2	4	22 (18-36)	100
CaCl <sub>2</sub> plus epinephrine	40 + .01 mgm	1	1	17	100
Digitalis group					
Digitalis	20.0 mgm.	1	3	22 (15-20)	100
Strophanthus	0.2 mgm	1	5	13 (0-19)	100
Strophanthin	0.01 mgm	2	2	23 (21-25)	50
Strophanthin	0.05 mgm	4	12	21 (3-33)	92

TABLE 1—Continued

AGENT	DOSE PER KGM.	NUMBER OF ANIMALS	NUMBER OF TRIALS	AVERAGE PER CENT FALL OF BLOOD PRESSURE AND RANGE	COMPLETE RECOVERY PER CENT OF TRIALS <sup>§</sup>
Xanthines					
Caffeine	10 mgm	4	7	28 (24-32)	72
Aminophylline	5-30 mgm	3	5	55 (29-83)	60
Metabolic stimulants					
Dinitrophenol†	10 mgm	1	1	24 (18-29)	0
Thyroxin†	1 mgm	1	1	33 (6-49)	100
Reducing agents					
Sodium thiosulfate†	0.5-1.0 gm	3	4	37 (22-65)	50
Sodium formaldehyde sulfoxylate	1.0 gm	1	1	75 (50-100)	0
Vitamins, enzyme systems, and substrates					
Nicotinic acid†	50-100 mgm	3	7	18 (10-30)	43
Ascorbic acid†	50 mgm	4	4	22 (10-35)	40
Thiamin†	10 mgm	6	11	19 (2-63)	93
Riboflavin†	1 mgm	2	5	18 (9-20)	72
p-Aminobenzoic acid†	20-50 mgm	2	6	37 (23-48)	71
Sodium succinate†	100 mgm	4	5	80 (21-67)	78
Miscellaneous					
Methylene blue†	10-80 mgm	4	9	21 (0-36)	95
Guanidine	100 mgm	1	3	13 (10-16)	100
Ergotamine†	1 mgm	1	1	36	0
Physostigmine†	0.3-1 mgm	3	6	25 (11-42)	70
Nikethamide	10 mgm	3	6	54 (39-74)	33
Nikethamide	20 mgm	1	3	27 (17-35)	100

\* 30 mgm per kgm of quinine sulfate

† Administered prior to quinine

‡ Recovery of blood pressure to the level before injection of quinine

§ Numerals in parenthesis give the range

doses. The per cent of complete recoveries also tended to be of the same order for the different doses in the 2 states.

In general, a completely successful antagonist of the depression, resulting from doses of quinine of therapeutic order, in normal animals, should be capable of preventing or appreciably reducing a 30 per cent fall in blood pressure and producing recoveries of 75 to 100 per cent. These values are approximations derived from the control injections, and were used for estimating the value of the different agents tried. Two factors, which appeared obviously important, were used in assessing the efficiency of the different agents: 1) the average per cent fall of blood pressure, which reflected the degree of reduction or prevention of the depressor action, and 2) the per cent of complete recoveries, to the original level of blood pressure, in the total trials made. For example, when the average per cent fall in

blood pressure was zero there was complete antagonism of the depressor action of quinine and when the average per cent recovery was 100 the antagonism was definitely more complete. When the average fall in blood pressure was about one-half that of the control i.e. 15 or 13 per cent and the recovery 100 per cent this was accepted as being favorable to the antagonist. But when the average fall in blood pressure was over 30 per cent (control 29 per cent) and the average per cent recovery below 75 per cent the antagonistic efficiency was definitely unfavorable. It seemed clear then the lower the average per cent fall in blood pressure (definitely less than 30 per cent) and the closer the approach to 100 per cent of trials giving complete recovery the higher was the order of antagonistic efficiency of an agent and the smaller the hazard of intravenous injection of quinine. Comparisons of the results

TABLE 2

*Effects of quinine sulfate with colloidal agents injected intravenously in rabbits with abnormally low blood pressure (below 60 mm. Hg) produced by hemorrhage*

AGENT	DOSE	NUMBER OF ANIMALS	NUMBER OF TRIALS	AVERAGE PER CENT FALL OF BLOOD PRESSURE AND RANGE	COMPLETE RECOVERY PER CENT OF TRIALS†
10 mgm. of quinine sulfate per kgm.‡					
Saline solution (control)	8 cc./kgm.	5	13	30 (16-51)	77
Human plasma	8	2	8	45 (17-72)	50
Methocel 0.075%	8	1	1	0	
Acacia 6%	8	1	1	40	100
Pectin 3%	8	1	3	18 (17-20)	100
30 mgm. of quinine sulfate per kgm.‡					
Saline solution (control)	24	4	7	52 (23-83)	71
Human plasma	24	1	2	73 (71-75)	50
Methocel 0.075%	24	2	2	55 (28-85)	50
Pectin 3%	24	1	1	55	100
Congo red 1% in 5% dextrose	5	4	2	68 (46-90)	50

Numbers in parenthesis give the range

† Recovery to level before injection of quinine

‡ Quinine was administered with the different colloids except congo red (30 mgm. per kgm.) which was given prior to injection of the alkaloid

with the different agents used with quinine and quinine in saline solution (table 1) showed that about 3 agents satisfied these criteria of beneficial actions to some extent at least.

**COLLOIDAL AGENTS.** In the early South Pacific operations quinine in human plasma is reported to have been used intravenously with success in treating emergency cases with severe malarial infections. Whether this was done to mitigate the cardiac depression of the alkaloid or to promote general recovery of poor circulatory risks or both is not known. It is conceivable that the colloidal character of the plasma acting as a physical protective might reduce the intensity of the direct cardio poisoning by the alkaloid. Human citrated plasma was found to be practically innocuous for rabbits but it did not reduce the depressant action of quinine (table 1). On the contrary it tended to prolong recovery of the blood pressure. The same was true for methocel 0.075

per cent, pectin 3 per cent and 6 per cent, and acacia, 16 per cent, containing the quinine, and congo red, 50 mgm per kgm, injected prior to the quinine owing to precipitation when mixed with the alkaloid. The only 2 colloidal agents which showed partial benefits were acacia, 6 per cent, and pectin, 0.5 per cent and 6 per cent, containing the quinine. The degree of depressor action was practically not reduced, but the recoveries were complete. The viscosity of these 2 solutions is about the same as that of plasma. The higher dose of quinine, or 30 mgm per kgm, was only partially antagonized by pectin, 3 per cent, and acacia, 6 per cent, but not by the other colloids tried.

It seems that there is a limit to the antagonistic or protective efficiency of colloidal agents generally. Only pectin and acacia in lower concentrations indicate possibilities for mitigating the quinine depression and promoting recoveries. Both these agents have been used in clinical intravenous injections for shock with variable success. As compared with other agents to be described, acacia and pectin are definitely less desirable and not to be recommended. Besides the objectionable reactions to these agents, the solutions do not keep well and require special precautions in preparing and handling. Our result with acacia that it does not decrease the depressor action of quinine intravenously confirms an earlier observation by McCarrison and Cornwall (9).

**SYMPATHOMIMETIC AMINES** Injected together with the standard dose of quinine, epinephrine was the only one of 4 amines tried which acted beneficially in doses which might be permissible clinically. The doses used were 0.01, 0.015 and 0.02 mgm per kgm. The smallest dose was the least effective and most irregular. The following results were obtained in 6 otherwise untreated rabbits. In 2 rabbits, 0.01 mgm per kgm caused rises of blood pressure during the first 2 injections, but not the third injection in 1 animal, and still produced a rise during the third injection in another. In 2 other rabbits, this dose failed to produce rises of blood pressure during the first injections. The 2 higher doses definitely decreased and frequently prevented the depressor action. In fact, these doses mixed with quinine caused moderate and variable pressor actions during the first 5 injections in 3 rabbits and during the tenth injection in another rabbit, but the pressor action failed to occur during the fifth and sixth injections in 2 other rabbits, the previous doses having been effective. Typical results with quinine and epinephrine in different doses in otherwise untreated rabbits are illustrated in figure 1.

In 16 different rabbits, the majority of which received epinephrine and some other agents at some time, the average results showed a mitigation of the depressor action, i.e., 13 and 17 per cent reduction in blood pressure with 0.015 and 0.02 mgm per kgm doses of epinephrine, respectively, or roughly one half the fall in blood pressure in the controls injected with saline solution. Complete recoveries occurred in practically all of the 50 trials made. In many individual trials, the antagonism of epinephrine was actually better than the averages indicated, because there was complete prevention of the depressor action, as indicated by the ranges from zero upwards (table 1). The depressor action of quinine generally manifested itself after about the fourth or fifth injection of quinine-epinephrine, and this became progressively more marked as

the number of injections increased. From this it appeared that the heart could only sustain the increase in peripheral resistance caused by epinephrine until the organ was weakened by the quinine. Injections of epinephrine following the quinine were never as beneficial as mixtures of the 2 alkaloids. The heart was slowed at all times regardless of the anti-depressor action of the epinephrine. This did not mean that quinine paralyzed the sympathetic innervation to the heart because vasoconstriction was present at the same time with the bradycardia. Presumably the cardiac muscle itself was relatively more poisoned by the quinine than were the blood vessels. Our results in rabbits and some in cats (fig 3) agreed with those of Konzett (10) who used cats and found that the depressor action was prevented when the epinephrine was injected together with quinine.

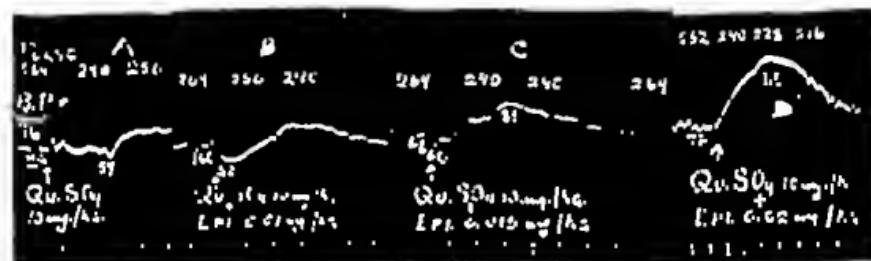


FIG 1 TYPICAL COMPARATIVE INTRAVENOUS EFFECTS OF EPINEPHRINE 0.01 0.015 AND 0.02 MGm PER KGM EACH WITH QUININE SULFATE 10 MGm PER KGM AND QUININE SULFATE ALONE ON BLOOD PRESSURE AND PULSE RATE IN RABBITS

Rabbit (2.47 kgm) (expt 78) A quinine sulfate (control) B, quinine sulfate with epinephrine 0.01 mgm and C quinine sulfate with epinephrine 0.015 mgm. Rabbit (2.54 kgm) (expt 64) D quinine sulfate with epinephrine 0.02 mgm.

Time mark 20 seconds also zero level for blood pressure (B.P.) Qu. S0<sub>4</sub> = quinine sulfate

These results with epinephrine-quinine mixtures sustain certain clinical recommendations for preventing or at least mitigating, the circulatory depression. However this practice would be expected to succeed with about 3 successive injections. Fortunately not more than 2 or 3 such injections of quinine with epinephrine in 24 hours spaced at 8-hour intervals would generally be required in antimalarial treatment. The higher doses used by us namely 0.015 and 0.02 mgm per kgm would be the equivalent of total doses of 1.05 and 1.4 mgm respectively for a 70 kgm adult man assuming a similar action. A dose of 1 mgm epinephrine which is close to the equivalent of 1.05 mgm tried by us in animals mixed with 0.5 gm quinine hydrochloride in 250 cc of saline solution and injected in 30 minutes has been used successfully by Escher and Villequez in man (8) and therefore experimental and clinical results agree closely. Accordingly it is believed that Escher and Villequez's recommendation is worthy of further clinical trial.

As for slow injections of quinine such as 20 or 30 minutes generally recommended clinically as compared with the rapid injection (2 minutes) used by us in animals this was tested in rabbits. The average per cent fall in blood pressure resulting from 2 injections of the standard dose of quinine sulfate alone for 17 and 20 minutes was practically the same or about 25 per cent as the average fall in more rapid injections (table 1). Two injections of epinephrine, in doses of 0.015 and 0.02 mgm per kgm prevented the depressor action and caused

increases in blood pressure of 7 and 20 per cent, respectively. While the number of animals and injections was too small to permit definite conclusions, the results obtained with long injections agreed with the much more rapid injections. The matter was not pursued further, since long injections were too time-consuming, and the short injections satisfied our purpose and permitted exploration of many other possible antagonists, besides epinephrine, and other aspects of quinine action.

Although the trials with neosynephrine were much more limited, the dosage necessary for antagonism of the quinine depression was much greater. Doses of 0.05 mgm per kgm definitely reduced the depressor action and improved the recovery as compared with saline solution. Doses of 0.1 mgm per kgm were somewhat less effective in reducing the depressor action, but produced complete recoveries, comparable to epinephrine. This dose would be the equivalent of 7 mgm for a 70-kgm adult, which is too large for safety, but the smaller dose (0.05 mgm per kgm) would give 3.5 mgm, which might be permissible. The situation was less promising with ephedrine, and the dosage was still higher. Doses of 5 mgm per kgm did not sufficiently prevent the depression, but recoveries in blood pressure to the original level occurred in all cases. Doses of 10 and 15 mgm per kgm were somewhat more beneficial, although definitely less than epinephrine, but all the doses of ephedrine tried would be prohibitive for man. In several trials, ephedrine markedly increased the depression after several doses of quinine had been injected and the heart became irregular and arrhythmic. These latter actions agreed with the well known poisoning effect of ephedrine on the heart. Therefore, this amine would not be clinically desirable. Benzedrine, in the few trials made, also proved comparatively ineffective or undesirable. Therefore, this leaves epinephrine as the sympathomimetic amine of choice for controlling, preventing, or mitigating the quinine depression. The counteraction of the immediate and sharp fall of blood pressure by epinephrine appears actually more satisfactory than an older impression given by McCarrison and Cornwall (9).

**CALCIUM** Calcium chloride was tried because of the stimulant action of small doses for the circulation. Doses of 10 mgm per kgm, injected together with the standard dose of quinine were found to be definitely beneficial, being comparable in degree to epinephrine in doses of 0.015 to 0.02 mgm per kgm. The depressor action was reduced 16 per cent, and complete recoveries occurred in 100 per cent of trials (table 1). Doses of from 20 to 80 mgm per kgm injected mixed with, or prior to, the quinine were less antagonistic than the smallest dose of calcium as the depressor action remained practically unchanged or was greater and recoveries were less complete. Transferred to a 70-kgm adult man, the 10 mgm dose of calcium chloride would give a total dose of 0.7 gm which might be used safely, if injected slowly. Theoretically, calcium lactate would be expected to be about as effective as the chloride, but our results with an equimolar solution and the same amounts of calcium ion showed it to be less, since the recovery was incomplete, and therefore, this salt is less desirable. Furthermore, solutions of calcium lactate are not as stable as those of calcium

chloride. Mixtures of calcium chloride in different doses with quinine and epinephrine 0.01 mgm. per kgm. were not more beneficial than epinephrine alone in different doses (table 1). There seemed to be no potentiated or additive actions of the calcium.

**OTHER AGENTS.** Six other general types of agents tried proved disappointing since the 2 criteria of beneficial action used were not generally satisfied at the same time. That is either the depressor action remained unchanged from the control or was aggravated and complete recoveries were less frequent when the depression was less, or they were occasionally more frequent when the depression was worse. The results (table 1) with these agents do not merit extended discussion except to summarize briefly the reasons for trying these.

The digitalis group was tried because of the demonstrated antagonism of quinidine for the early toxic circulatory manifestations of digitalis (11). It was postulated that conversely digitalis might antagonize the depressor action of quinine but this group proved comparatively inadequate and undesirable. The xanthines were used as possible coronary vasodilators and as direct cardiac stimulants and because these are recommended as resuscitative agents in quinine-collapse but they were not better or worse than saline solution. Since the fundamental cause of cardiac depression by quinine is a disturbed coordination of enzyme processes resulting from inhibition depression or paralysis of enzymes certain vitamins which belong to enzyme systems were tried in the sense of substitution therapy. This was the reason for trying riboflavin, thiamine and nicotinic acid. Ascorbic acid was tried because of its reputed antitoxic actions for certain vascular effects. However its reducing action would not necessarily serve our purpose. Para-aminobenzoic acid was also tried on the general principle of an antitoxic agent. Methylene blue is an oxidation reduction dye which can function in tissues as a respiratory catalyst and thus permit cellular activity after paralysis of natural catalysts. Its action in this respect would be most efficient in rodents in which its action as a methemoglobiniser is least efficient. In rabbits it had some antagonistic action to quinine chiefly as to recovery from the depressor action. In other mammals including man its power of methemoglobin formation would be undesirable in cardiac depression. Succinate was tried on the assumption that it might continue to be utilized as a substrate by the quinine poisoned heart and thus sustain the functional activity of this organ. This was postulated on certain results with quinine reported by Wright and Sabina (12) and on Holmes (13) statement that the heart contains much cytochrome which is very active in oxidising succinate. However no vitamin catalyst or substrate was sufficiently beneficial to be of practical usefulness in immediate or emergency states except possibly thiamine whose antagonistic efficiency was similar to that of calcium chloride 10 mgm. per kgm. or epinephrine 0.02 mgm. per kgm. However longer periods of treatment with thiamine such as those used by Govier and Greer for shock and hemorrhage (14) and which might result in some intrinsic antagonism of quinine depression was regarded as not coming within the scope of our objective.

Trials with the metabolic stimulants dinitrophenol (injected intravenously) and thyroxin (given hypodermically 24 hours before quinine) might be classed with the catalyst-category. Both drugs increased the body temperature but no worthy antagonism occurred and even if it had these agents would not be desirable. The reducing agents, sodium thiosulfate and sodium formaldehyde sulfoxylate were tried on the general principle of antidotal action but they aggravated the depression. The same occurred with ergotamine and physostigmine (peripheral vasoconstrictors) and nikethamide (vasomotor center stimulant and recommended resuscitative agent). Three injections of guanidine (peripheral vasoconstrictor) with quinine in 1 rabbit were partly antagonistic but the high dosage of guanidine would be undesirable clinically.

**TOXICITY OF QUININE INTRAVENOUSLY IN TREATMENTS WITH DIFFERENT TYPES OF AGENTS.** It might be of practical importance to know whether the agents used for tests of antag-

omistic action of the quinine-depression affected the toxicity of quinine. An antagonist might be temporarily beneficial, i.e., prevent or mitigate the depression of 1, 2 or 3 therapeutic doses of quinine, but further use of the antagonist might aggravate it, reduce the total dose of quinine which could be tolerated, and result in death. Again, the continued use of an antagonist, with quinine, might not affect the total amount of quinine that would be fatal without the antagonist. The majority of the agents tried (tables 1 and 2) possessed definite actions with more or less specific effects on physiological functions. Therefore, the cumulative effects might be disturbing to the system, and the comparative results might shed some light on the possible hazards attached to the intravenous use of quinine under these or similar conditions.

The total doses of quinine sulfate resulting from repeated intravenous injections of the standard dose until death were tabulated according to selected, predominant types of agents as used with quinine (table 1). Some of these might actually be used with quinine, or separately, in medication for some other condition besides malaria. Table 3 presents a list of such average fatal doses of quinine arranged in descending order, using saline solu-

TABLE 3

*Intravenous fatal doses of quinine in rabbits treated with different types of agents\**

PREDOMINANT TYPE OF AGENT	AVERAGE FATAL DOSE OF QUININE mgm per kgm
Physiologic salt solution (control) (5)	200
Vitamins, etc (4)	243
Sympathomimetic amines (5)†	220
Colloids, all (11)	200
Human plasma (5)	210
Other colloids (6)	135
Calcium chloride (6)	190
Digitalis group (3)	160

\* Median initial blood pressure in all rabbits was 80 mm Hg, range 60 to 95 mm Hg. The number of rabbits compared is indicated in parenthesis.

† Almost entirely epinephrine and norepinephrine.

tion as control for comparison. Only those rabbits were used in which at least 3 animals had been injected with the predominant type of agent, which, in most cases, was the exclusive agent.

Although the number of animals in which the comparisons could be made was small, the general trend of toxicity for quinine according to type of agent was suggestive. That is, the highest average fatal dose, or 200 mgm per kgm, occurred in animals receiving the alkaloid in physiological salt solution only. The least objectionable and most favorable antagonists, namely, the vitamins and sympathomimetic agents, came next in order with average fatal doses of 243 mgm and 220 mgm per kgm, respectively. Collectively, the colloids were unsatisfactory as antagonists and tended to maintain the depressor action, and here, the average fatal dose was appreciably less, i.e., 200 mgm per kgm, various foreign colloids giving a lower average fatal dose, or 135 mgm per kgm, than plasma, which gave 210 mgm per kgm. It is seen that from here on the average fatal doses were progressively smaller, than for the other agents, i.e., 190 mgm per kgm for calcium chloride, and 160 mgm per kgm for the digitalis group. No agent tried raised the fatal dose.

Thus, in general, the least favorable antagonists and the most undesirable agents for the depressor action of quinine in doses of therapeutic order (table 1) agreed with an increased toxicity of the alkaloid according to the estimates made. It would seem that quinine used repeatedly might not be as well tolerated in individuals receiving, also re-

peatedly agents of this kind such as foreign colloids, calcium and digitals. These results in animals are only suggestive because of the high and frequent dosage of these agents and quinine which would not be used clinically. However man is generally more sensitive and illness may be a contributory factor and therefore in principle at least cautious use of these combinations would be leaning to the side of safety. On the other hand the hazards would appear to be definitely less in using similarly vitamins or epinephrine and neosynephrine with quinine. This might be expected because vitamins are practically devoid of acute toxicity and these sympathomimetic amines tend to be rapidly destroyed or their effects are not sufficiently cumulative. The consideration of these various possibilities is not to be confused with the beneficial effects of a single dose of epinephrine or neosynephrine with a single therapeutic dose of quinine.

**THE CIRCULATORY DEPRESSION OF QUININE AS A CHOLINERGIC PHENOMENON**

Waelsch and Nachmansohn (15) reported recently that quinine and quinacrine (atahrine) inhibit cholinesterase *in vitro* quinacrine being 100 to 200 times more efficient according to molar concentrations. On the basis of these results these authors suggested that certain peripheral toxic manifestations of quinacrine including vagal stimulation are due partly to inhibition of cholinesterase. This would presumably include the circulatory manifestations, which are the same for both drugs. We were interested in determining the validity of this theory for the circulatory depression of quinine even though it is weaker than quinacrine *in vitro*. For if the postulated correlation were true for the living organism, the explanation of the quinine-depression would be relatively simple and a typical reversal of this action by atropine would also provide a measure for preventing or correcting the depression.

Using 4 rabbits and 6 cats, with a highly sensitive tambour recording on a kymograph by air transmission from an oncometer which enclosed an entire hind leg together with the usual records of blood pressure and pulse rate changes for quinine the typical circulatory effects of acetylcholine or mecholyl were demonstrated and also the reversal of their effects by atropinization. However the same animals injected intravenously with 5 and 10 mgm per kgm doses of quinine bisulfate (to permit using the smallest possible volume of saline solution) before or after the cholines and also independently of the cholines in completely atropinized animals (abolition of cardiac slowing and fall of blood pressure during electrical vagus nerve stimulation) reacted invariably unchanged from the action of quinine alone. That is the bradycardia, the depressor effect and a small immediate and fleeting peripheral vasodilatation persisted in animals typically reactive to the cholines and reversible by atropine. Curarization did not affect the results. Therefore the cholinergic theory of the circulatory depression of quinine lacks validity and by the same token atropine would be of no value in preventing or checking the depression or collapse. Figure 2 illustrates typical results obtained with quinine.

**THE PERIPHERAL VASODILATATION OF QUININE.** It has long been known that quinine causes a peripheral vasodilatation apparently mainly in the skin (16). However the nature and importance of this in the depressor action of quinine has not been entirely clarified. In our tests for the implied cholinergic action of the alkaloid the vasodilator action was demonstrated in the peripheral vessels

that is, in the hind legs, but this did not establish the seat of the action, which might be in the vasomotor center, the subsidiary centers in the spinal cord, or in the blood vessels. Therefore, 5 other cats were prepared as for the cholinergic tests, except that both hind legs were put into oncometers which were connected with a single tambour, and, at the same time, onometric records were made of different viscera in different animals. The results were strikingly conclusive for the seat of quinine-action in the peripheral blood vessels, because the leg oncometers recorded a sharp and marked increase in volume with simultaneous passive constriction of a kidney or intestine, before and after section of the

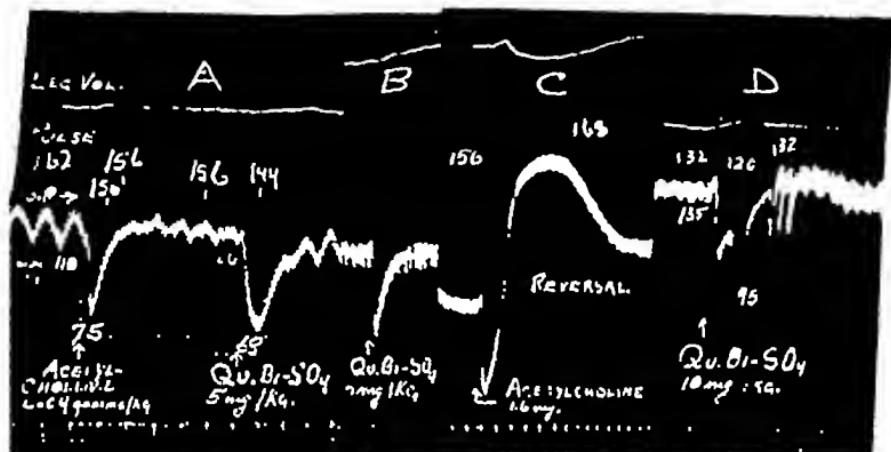


FIG. 2 TYPICAL VASOMOTOR REACTIONS TO ACETYLCHOLINE, INCLUDING VASOMOTOR REVERSAL AFTER ATROPINIZATION, WITH THE TYPICAL DEPRESSOR ACTION OF QUININE INJECTED INTRAVENOUSLY BEFORE AND AFTER ATROPINIZATION IN CATS

Cat (2.96 kgm) (expt 57) A, acetylcholine 0.04 gamma per kgm and quinine bisulfate 5 mgm per kgm, as controls, B, quinine bisulfate, same dose, after atropinization (2 mgm per kgm atropine intravenously, vagi paralyzed, leg volume somewhat exaggerated), C, vasomotor reversal (rise of blood pressure and vasoconstriction in leg) after acetylcholine 1.6 mgm.

Cat (4.21 kgm) (expt 62) D, quinine bisulfate 10 mgm per kgm, caused the usual fall of blood pressure and vasodilation in the leg, preceded by complete atropinization, vasomotor reversal of acetylcholine and usual control effects from quinine and acetylcholine before atropine, as in Expt 57, above, the latter changes not being shown in this figure.

Time mark 20 seconds, also zero level for blood pressure (B.P.), one hind leg in an onometer (see greater leg volume changes, using 2 legs in fig. 3). All drugs were injected intravenously.

cervical cord and destruction of the spinal cord by pithing. The doses of quinine injected intravenously were 5 and 10 mgm per kgm, the responses being generally smaller with the higher dose, though not always. Typical results are illustrated in figure 3. The peripheral vasodilatation occurred during the fall of blood pressure and the cardiac slowing, and sometimes recovered before the depressor action and bradycardia recovered. But, when the latter were marked and sustained, as with higher doses or repeated small doses of quinine, the onometric record showed a reduction in volume, as the result of cardiac depression.

Since the typical vasodilatation occurred in atropinized animals, and was not reversed to vasoconstriction, it was not mediated by a cholinergic action.

It was not due to depression or paralysis of sympathetic innervation because injection of epinephrine promptly reversed the vasodilatation to vasoconstriction contrary to Nelson's view (16). The vasodilator action was generally not demonstrable after the third or fourth injection of quinine. Whether this was due to tachyphylaxis in blood vessels was not investigated but the heart continued to be depressed. It follows therefore that the vasodilatation of quinine injected intravenously is an early phenomenon which is not only comparatively minor and evanescent and of little or no importance in the typical

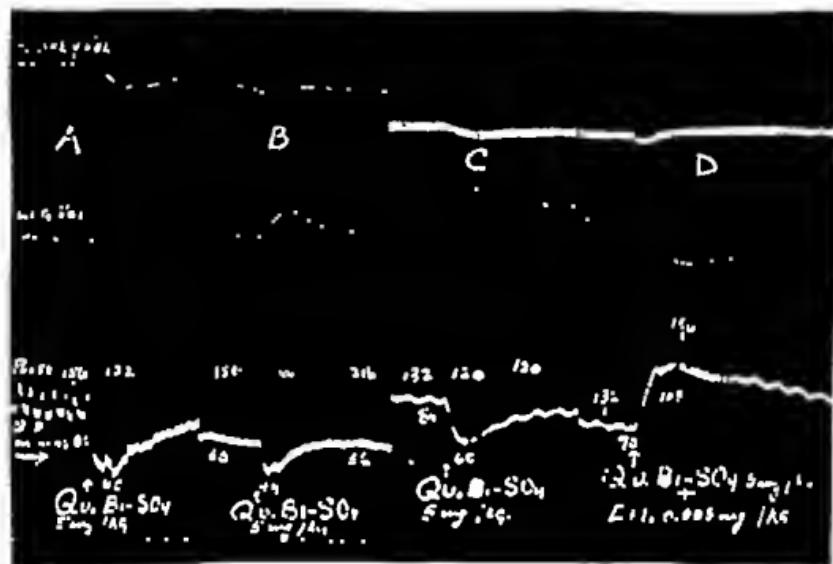


FIG. 3. TYPICAL PERIPHERAL VASODILATATION IN THE HIND LEGS AND PASSIVE CONSTRUCTION IN A KIDNEY AFTER INTRAVENOUS INJECTION OF QUININE BISULFATE, 5 MG/M PER KGM. IN A CAT (3.04 KGM.) (EXPT 90) AFTER SECTION OF THE CERVICAL CORD (A) AND PITHING OF THE SPINAL CORD (B), AND SIMILAR VASOMOTOR CHANGES WITH DEPRESSOR ACTION IN ANOTHER CAT (2.94 KGM.) (EXPT 85) WITH CERVICAL CORD CUT AFTER QUININE BISULFATE ALONE (C) AND ANTAGONISM OF THE VASODILATATION IN THE LEGS BY EPINEPHRINE 0.005 MG/M PER KGM. MIXED WITH THE SAME DOSE OF QUININE IN THE SAME CAT (D).

Time-mark 20 seconds also zero level for blood pressure (B.P.) Qu. Bi-SO<sub>4</sub> = quinine bisulfate leg volume from both hind legs together

sustained depressor action but it is also not of central, cardiac or cholinergic origin.

VALUE OF RESUSCITATIVE AGENTS IN QUININE-COLLAPSE. Epinephrine, caffeine, aminophylline and nikethamide (coramine) are recommended as resuscitative agents, the last 3 agents being advised in certain military directives. Epinephrine is probably more relied upon in civilian practice so that this drug is recommended to be kept ready at hand to combat the sudden and alarming depressor effects of quinine intravenously. As already discussed in the forepart of this paper some clinicians advise giving epinephrine with quinine a practice which is supported by our experimental results in animals which could be classed as good circulatory risks (normal or high blood pressure). However

resuscitative agents are used or recommended in sudden circulatory collapse when the blood pressure falls to the shock, or below shock, level and the heart beat is barely perceptible. Such severe reactions are also reported for ordinary clinical intravenous doses, probably when injections are made too rapidly, or when the concentration of quinine in solution injected is too high.

The following resuscitative agents were tried by us when the blood pressure generally reached a level of 20 mm Hg or less: epinephrine, nesynephrine, ephedrine, caffeine, aminophylline, nikethamide, and 0.9 per cent sodium chloride solution, as control. The median level of all blood pressures was 7 mm Hg (range, 3 to 20 mm Hg). The only exception was 1 animal used at a level of 50 mm Hg with aminophylline. With the most promising agents, 3 different doses were tried, with others, only 2. The 2 chief criteria used were the degree of sustained recovery in blood pressure, usually for 10 minutes, estimated in per cent of the initial level of blood pressure, and the per cent of trials giving the sustained recovery. The immediate and evanescent peak pressor effects of the sympathomimetic amines were not considered, as it was believed that the sustained recovery was a more desirable objective. Our criteria were more indicative of an immediate and relatively short-lasting resuscitative effectiveness than a permanent recovery. However, it is probable that, once sustained recovery occurred, this would have been permanent in many, if not the majority, of the animals, which were not observed further, but used for other tests, as their circulation was in reasonably good condition. The essential data are presented in table 4.

Using average values for estimating the comparative efficiency of the different agents tried, it is clear that caffeine, 5 and 10 mgm per kgm, aminophylline, 10 mgm per kgm, ephedrine, 5 and 10 mg per kgm, and neosynephrine, 0.1 and 0.3 mgm per kgm, were practically valueless, and not much better, or worse, than saline solution, alone. Caffeine and aminophylline caused a further fall of blood pressure in about one-half of the trials and animals. In the higher dose, or 30 mgm per kgm, aminophylline caused a recovery about equal to epinephrine, 0.05 mgm per kgm, but this dose of aminophylline would be prohibitive in man. Caffeine is generally given hypodermically, while aminophylline and nikethamide are given intravenously in emergencies, but for purposes of comparison, all drugs were given by us intravenously, i.e., under the same conditions. The use of saline solution was generally combined with cardiac massage by compression of the chest, and artificial respiration, but to no avail in the majority of trials and animals. In the 10 rabbits used, the sustained recovery was only 10 per cent in 23 per cent of trials. Cardiac massage raised the blood pressure mechanically during the compressions, but this fleeting pressor action was omitted in estimating the value of saline solution alone. Of the remaining agents, epinephrine, in doses of from 0.02 to 0.1 mgm per kgm, came first, nesynephrine, 0.05 mgm per kgm, was a close second, and nikethamide, 10 and 20 mgm per kgm, third, in order, according to efficiency and desirability. However, the resuscitative efficiency, under our conditions, should be regarded as not more than trends because there were individual animals

which did not recover after treatment with all these agents as indicated by the ranges in the results from zero upwards. The number of animals was not uniform throughout, and with some the number of doses was too small to permit fine distinctions. However the high resuscitative efficiency of epinephrine in the smallest dose tried or 0.02 mgm per kgm agreed with the generally beneficial effects of the same dose of epinephrine on the depressor action of quinine in

TABLE 4

RESUSCITATIVE AGENT	NUMBER OF ANIMALS	NUMBER OF TRIALS	AVERAGE INITIAL BLOOD PRESSURE AND RANGE	AVERAGE QUININE-COLLAPSE LEVEL OF BLOOD PRESSURE AND RANGE	RESUSCITATIVE AGENT*		PER CENT OF TRIALS GIVING SUB-TAILED RECOVERY
					Dose	Average per cent stabilized recovery of initial blood pressure and range	
Physiologic saline solution (control)	10	13	mm. Hg 73 (60-90)†	mm. Hg 7 (0-16)†	6 cc	10 (0-70)†	23
Epinephrine	5	8	82 (64-100)	8 (5-10)	0.02	55 (0-100)	75
	11	14	65 (60-84)	6 (0-20)	0.05	38 (0-118)	26
	6	8	59 (30-95)	5 (0-10)	0.10	24 (0-100)	33
Neosynephrine	6	8	76 (60-85)	10 (2-20)	0.05	38 (0-100)	50
	11	13	75 (50-95)	7 (0-30)	0.10	10 (0-125)	23
	1	1	95	5	0.30	0	0
Nikethamide	5	6	111 (70-140)	7 (0-10)	10.0	27 (0-127)	33
	2	5	90 (70-110)	3 (0-10)	20.0	44 (0-93)	60
Aminophylline	1	1	85	50	10.0	0	0
	2	4	62 (44-80)	10 (5-15)	30.0	36 (68-75)	50
Caffeine	2	2	71 (60-92)	10 (10-15)	5.0	0	0
	5	7	70 (50-83)	20 (0-35)	10.0	1 (0-11)	0
Ephedrine	1	1	90	5	5.0	0	0
	3	3	78 (60-90)	8 (5-15)	10.0	0	0

Injected at time of collapse (column 5)

† Numerals in parenthesis give the range

animals with high blood pressure already discussed. Neosynephrine and epinephrine in 2½ times the smallest dose of epinephrine were about equally satisfactory, as indicated by the nearly identical per cent recoveries of the initial blood pressure in a similar per cent of trials. Nikethamide in only the smaller dose of 10 mgm per kgm would probably be permissible clinically but this caused only a recovery of 27 per cent in the blood pressure as against 55 and 38 per cent produced by epinephrine 0.02 and 0.05 mgm per kgm respectively, and 36 per cent by neosynephrine 0.05 mgm per kgm. The higher dose of nikethamide or 20 mgm per kgm would not be permissible clinically owing

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# BEHAVIOR IN THE BODY OF SOME FRACTIONS OF GELATIN<sup>1</sup>

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Since the recent revival of interest in gelatin as a blood substitute (1-2) several studies of the pharmacological properties of gelatin solutions in experimental animals and in man have been made (3-8). It has been reported that gelatin, after intravenous injection into normal animals and men remains in the blood stream for some time, retaining fluid and thus increasing the plasma volume. Desirable therapeutic results from the administration of gelatin solutions have been described in dogs following hemorrhage, burns and other trauma and in patients suffering from a variety of conditions of shock.

Gelatins obtained from collagens of different sources, as bone and skin have been used (3) but many of the investigations have been with a particular bone gelatin.<sup>2</sup> Gelatin is recognized to consist of molecules of varying molecular weight (9, 10) and attempts have been made (11) to determine the relation of therapeutic value to molecular size by studying solutions of gelatin held at high temperatures for varying lengths of time to produce varying degrees of hydrolysis. It appeared to us that the preparation of fractions of molecular weight varying from high to low might be preferable to the use of graded proteolytic degradation. Such fractions have been prepared from the above mentioned gelatin<sup>2</sup>, characterized and studied.

**PREPARATION AND PROPERTIES OF GELATIN FRACTIONS.** The whole gelatin studied and used for fractionation was calcium gelatinate produced by hydrolysis of alkali treated bovine long bone collagen supplied by the Knox Gelatine Co. and identified as B78-1. It was fractionated by alcoholic precipitation (12). Essentially the highest molecular weight fraction was precipitated originally at 23°C at a concentration of 55% alcohol. After repeated reprecipitation it was finally precipitated at 0°C in 50% alcohol. The middle molecular weight fraction was precipitated at 20°C in 47.5% alcohol and the lowest molecular weight fraction precipitated at 0°C in 47.5% alcohol. The colloidal osmotic pressure exerted by solutions of these fractions in 0.9% NaCl adjusted to pH 7.3-7.5 was measured at 37° in a Hepp osmometer (13) with No. 300 cellophane (fig 1). The average molecular weights were calculated from the classical equation

$$M = RT \frac{C}{P}$$

where  $C$  = grams of gelatin per liter of solution  $P$  = osmotic pressure in atmospheres  $R$  = 0.082 liter-atmospheres per degree  $T$  = absolute temperature. Since this equation is accurate only for very dilute solutions the  $C/P$  values at infinite dilution were obtained by extrapolation of the data in figure 1 plotting  $C/P$  against  $C$  and using the method of least squares assuming a linear relationship. The density and viscosity of these solutions

<sup>1</sup> This investigation has been aided in part by a grant from the Knox Gelatine Company

<sup>2</sup> Gelatin B78-1 Knox Gelatine Co.

at 37° were measured with a pycnometer and an Ostwald viscometer with a 5 ml. bulb and a water-effluent time of 62 seconds (fig 1). Relative viscosity was calculated as  $\eta/\eta_s = p_t/p_s t_s$ , where  $p$  and  $p_s$  are the densities of solution and solvent, and  $t$  and  $t_s$  are the effluent times of solution and solvent. From these data, the intrinsic viscosity ( $H_0$ ) was calculated according to the following equation

$$H_0 = \lim_{C \rightarrow 0} \frac{2.3 \log \eta/\eta_s}{C}$$

where  $\eta/\eta_s$  is the viscosity of the gelatin solutions relative to the solvent,  $C$  is the concentration in grams of gelatin per 100 cc of solution. The data were extrapolated to infinite dilution, using the method of least squares, assuming a linear relationship. Intrinsic viscosity represents the rate of change of relative viscosity with concentration at infinite dilution. It is useful as a characterization since its value depends but little upon the

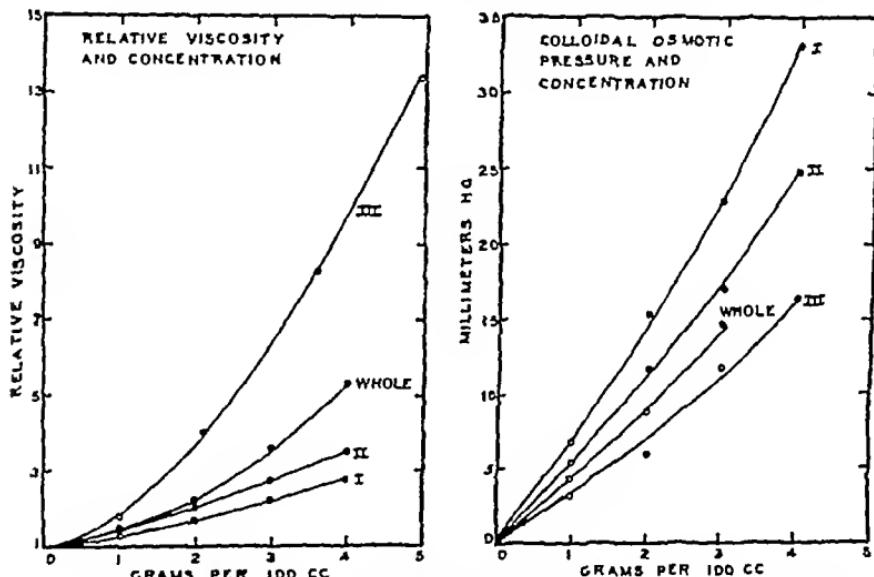


FIG. 1 THE RELATION OF RELATIVE VISCOSITY AND COLLOIDAL OSMOTIC PRESSURE TO THE CONCENTRATION OF SOLUTIONS OF GELATIN FRACTIONS

temperature and gelatin concentration (14). Water content was determined by drying to constant weight at 105°C. Nitrogen content was determined by a micro Kjeldahl method (15), glycine content (16), and hydroxyproline content (17) colorimetrically. None of the figures in table 1 are corrected for content of water since we are not confident that the figures in the table represent the true water content (18).

**EXPERIMENTS** Male dogs weighing between 13.3 and 21.7 kgm were used. They were anesthetized with ether long enough to permit exposure of a femoral vein and the intravenous injection of 250 mgm per kgm of sodium barbital. A period of three hours was allowed for approachment to a constant state (19). Blood was taken for determination of protein, CO<sub>2</sub>, and the hematocrit values. The plasma and erythrocyte volumes were then determined. The warmed gelatin solution was injected in a cephalic vein during 10 min units. The gelatin solutions were made in 0.9% NaCl in concentrations estimated to have colloidal osmotic pressures equivalent to that of dog's plasma as measured in our osmometer, = 20 mm Hg (See table 1, "isoosmotic concentration"). Twenty five cc per kg were given in each case. The pH of these solutions was not adjusted. The above men

tioned observations were repeated at 1 and 4 hours later. Carotid arterial pressure was measured with a Hg manometer throughout the experiment. The urinary bladder was emptied just before and 4 hours after the injection of gelatin. All blood samples were taken with a cannula in a branch of a femoral artery into tubes containing dried oxalates 1.8 mgm of ammonium and 0.7 of potassium per cc. In four experiments erythrocyte counts were made on the same samples of blood on which the hematocrit reading was determined. Two control experiments were done in one of which the blood drawn for samples was replaced by compatible blood from another dog.

**Methods** The plasma volume was determined with T 1824. Twenty mgm were injected for the control observation and 10 mgm at the 1 and 4 hour periods. Blood samples were taken at 20, 30 and 40 minutes later and the dye content of the diluted plasma measured in a Klett-Summerson photocomparator. The dye concentration at time of injection was obtained by extrapolation of a time log concentration curve. The total erythrocyte volume was measured with carbon monoxide measured into a bag-soda lime canister system with a burette. The purity of the CO was determined by analyzing for O<sub>2</sub> with the Van Slyke manometric apparatus and assuming the only contaminant to be air. Twenty minutes after applying the CO the blood CO content and capacity were determined in duplicate by a palladium method (20). The CO content of the gas in the lung-bag system was determined with the same method and the volume of gas in the bag measured. The dead space of the canister and tubes was found to be 550 cc by dilution with N<sub>2</sub> and analysis.

TABLE I

FRACTION	AVERAGE MOLECULAR WEIGHT	INTRINSIC VISCOSITY	WATER	NITROGEN	GLYCINE	ERYTHROCYTE FRACTION	ISOHEMOGLOBIN CONCENTRATION
			%	%	%	%	gm. per 100 cc.
Whole	50,000	0.39	11.91	15.80	26.8	8.17	3.90
I	29,000	0.30	14.06	15.70	26.2	7.97	2.60
II	36,000	0.33	8.73	16.49	26.8	7.90	3.25
III	69,000	0.65	7.54	16.00	25.2	8.29	4.85

Concentration at which colloidal osmotic pressure = 20 mm. Hg

sis. The lung volume as measured in a dead dog was taken to be 250 cc. A lung volume of 209 cc for dogs weighing 8-14 kgm has been reported (21). The hematocrit reading was determined in Wintrobe tubes for each sample of blood analyzed for CO. The values were corrected to 91.5% of the observed value (22). The plasma proteins were measured gravimetrically on 3 or 5 cc samples (23).

Studying the method of analysis for gelatin by difference between trichloroacetic and tungstic acid precipitation (1) we found that gelatin and plasma protein were precipitated by adding 1 cc of plasma to 9 cc of tungstic acid solution as used by Van Slyke and Hawkins (24) diluted with one-half volume of water. The lowest concentration of trichloroacetic acid at which the plasma proteins are precipitated was found to result from the addition of 1 cc of plasma to 9 cc of 5% trichloroacetic acid. Table 2 shows the results obtained with such procedures. Approximately 1% solutions of the gelatin fractions were made in blood serum. The total N and N of trichloroacetic and tungstic acid filtrates were determined for the serum and each solution in serum by a micro-Kjeldahl method (15). The factor 6.25 was used to convert N to protein and gelatin. The actual concentration of gelatin in each solution was determined by subtracting the total N content of the serum from the total N content of each solution of gelatin in serum and multiplying by 6.25. It appears that some gelatin is precipitated by the trichloroacetic acid. The error is largest for the high-molecular weight fraction and since these are the molecules apparently remaining longest in the circulation large errors might be expected as long as any gelatin

injected contains such molecules. Accordingly, we have determined gelatin as hydroxy proline (17), on 3 or 5 cc samples. Although our values for hydroxyproline content are lower than claimed (17), this has no influence on the value of the method, since the factor for calculation is as determined by us. Urine gelatin was measured by micro Kjeldahl determination of N in a recovered tungstic acid precipitate. Since normal dog urine gives a nitrogen-containing precipitate with tungstic acid (table 3), the gelatin figures are only to be compared for the fractions. Sedimentation of erythrocytes was observed in 15 cc of blood in centrifuge tubes, since irregular results were obtained after gelatin in Wintrobe tubes. The results were recorded as cc per minute, the maximum velocity being measured graphically. Duplicate determinations varied as much as 25%.

#### CALCULATIONS

$$V_{ET} = \frac{CO_{app} - CO_{rem}}{\left( \frac{CO_{blood}}{H} \right)_{after CO} - \left( \frac{CO_{blood}}{H} \right)_{before CO}} \text{ cc}$$

$$Hb_{circ} = \frac{CO_{cap} \times V_{ET}}{H \times 1.36} \text{ gm.}$$

$$Hb_{cap} = \frac{CO_{cap} \times 100}{H \times 1.36} \text{ gm}$$

$$MCHb = \frac{CO_{cap}}{E \times 10^7 \times 1.36} \text{ micromicrograms}$$

$$V_E = \frac{H}{E \times 10^7} \text{ cu. micra}$$

Where  $V_{ET}$  = total volume of circulating erythrocytes,  $CO_{app}$  = cc carbon monoxide placed in bag,  $CO_{rem}$  = cc carbon monoxide remaining in lung bag system,  $CO_{blood}$  = blood carbon monoxide content in cc per 100 cc,  $H$  = percentage of erythrocyte volume in hematocrit,  $Hb_{circ}$  = total circulating hemoglobin,  $Hb_{cap}$  = hemoglobin per 100 cc erythrocytes,  $CO_{cap}$  = carbon monoxide capacity, cc per 100 cc of blood,  $MCHb$  = mean corpuscular hemoglobin,  $E$  = number of erythrocytes per cu mm of blood,  $V_E$  = mean volume of a single erythrocyte.

#### DISCUSSION

The results appear in full in table 3, as averages in table 4.

The degree of retention of the different gelatins in the circulation is associated with, but is not proportional to, their molecular weight. If the percentage retention is plotted graphically against the average molecular weight (fig. 2), it is seen that a gelatin of average molecular weight of 25,000 or less can be expected to escape rapidly from the circulation, and that the percentage retention is not markedly increased when the average molecular weight goes above 50,000.

A similar situation is seen with retention of fluid, if the very small retention of experiment 13 is disregarded. The volume of fluid retained per gram of gelatin retained at 4 hours by the different fractions varied no more than among animals receiving the same fraction. The average for this value in all experiments was 28.1 cc per gm, which is larger than the value of 23 cc reported for human albumin in man (25).

The total plasma protein fell no more than in the control experiments, except in experiment 13. It is of interest that this animal also retained little of the

injected fluid. Others (4) found no change in total plasma protein when similar amounts of one of these gelatins (B78-1) were given. When 50 cc per kgm. of gelatin solution were given in an earlier study (8) a reduction in plasma protein was seen.

It has been reported (4) that the injection of gelatin solution into normal dogs causes a reduction in the volume of circulating erythrocytes, as calculated from the plasma volume and hematocrit reading. This method of calculation can be criticized on the basis that the hematocrit value is not the same throughout the circulation (20). We find no reduction in the total circulating hemoglobin except in one experiment with the whole gelatin and in the experiments with the high molecular weight fraction. In experiments in which there is an apparent reduction in total circulating hemoglobin there is a discrepancy among the mean corpuscular volume which decreases and the mean corpuscular hemoglobin which decreases, and the hemoglobin per 100 cc. of erythrocytes which also decreased. In the other experiments there was a decrease in mean corpuscular volume, no change in mean corpuscular hemoglobin, and an in-

TABLE 2

FRACTION	PLASMA PROTEIN	NONPROTEIN NITROGEN	GELATIN BY DIFFERENCE	GELATIN BY PRECIPITATION	PERCENTAGE ERROR	
					Plasma protein	Gelatin
Serum	5.49	0.025				
Whole	5.56	0.027				
I	5.40	0.028	0.93	0.85	+1.8	-10.5
II	5.48	0.027	1.00	0.96	0.0	-4.0
III	5.85	0.027	0.93	0.94	-1.1	-1.0
				0.56	+8.6	-41.0

\* Gelatin = 6.25 times difference in total nitrogen (see text)

crease in hemoglobin per 100 cc of erythrocytes all of which are explained by shrinkage of the cells. A possible explanation of the results of experiments 5 and 13 is that the gelatin had interfered with the combination of hemoglobin with carbon monoxide. A more likely explanation is that rapid sedimentation of the cells resulted in the introduction into the pipette of blood containing fewer cells than were present in the whole blood sample. This chiefly gives a low figure for carbon monoxide capacity which was low in these experiments and is the figure on which the mean corpuscular hemoglobin hemoglobin per 100 cc. of erythrocytes and total circulating hemoglobin largely depend.

In all experiments there is evident a reduction in the size of the erythrocytes. This has also been reported to follow the injection of blood serum into dogs (27). It is possible that in the experiments mentioned above (4) beyond the error of the method of calculating erythrocyte volume the shrinkage of the cells may account for the apparent decrease in total volume.

Sedimentation of erythrocytes was increased by all gelatin fractions. The acceleration of sedimentation was the same for the 20,000 and 36,000 molecular weight fractions at 1 and 4 hours and the 50,000 molecular weight gelatin at

4 hours. The acceleration was somewhat greater for the 50,000 molecular weight gelatin at 1 hour, and much greater for the 69,000 molecular weight gelatin at 1 and 4 hours. These comparisons are not essentially altered if the increase in sedimentation is calculated per gram-molecular concentration in plasma.

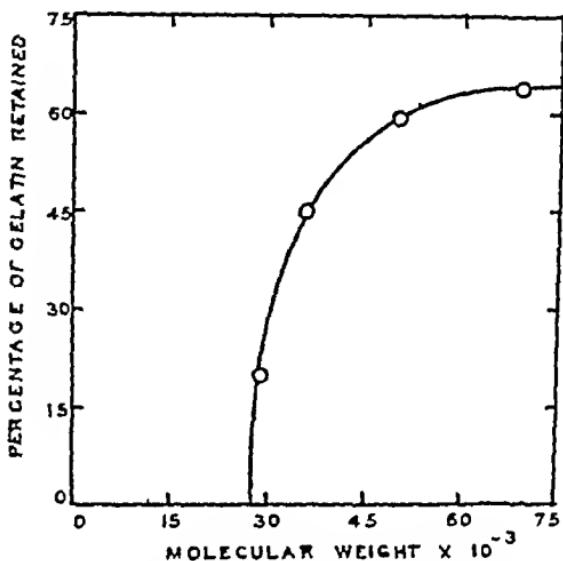


FIG. 2 THE RELATION OF RETENTION OF GELATIN IN THE CIRCULATION 4 HOURS AFTER INJECTION TO THE MOLECULAR WEIGHT

TABLE 4

GELATIN MOLECULAR WEIGHT	PERCENTAGE RETENTION				URINE GELA- TIN % EX- CRETED	PERCENTAGE OF CONTROL VALUE								
	Gelatin		Fluid			Plasma protein		Erythrocyte volume		Hemoglobin		Erythrocytes sedimentation		
	1 hr	4 hr	1 hr	4 hr		1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	
Control 1						87	73	94	88	95	97			
Control 2						95	90	105	105	103	111	75	106	
29,000	30	20	59	9	38.0	101	87	104	90	107	97	390	350	
36,000	52	45	54	38	12.2	88	88	102	91	113	101	377	358	
50,000	65	59	72	63	11.2	93	90	113	90	110	94	674	335	
69,000	80	64	82	42	4.9	87	83	73	82	68	100	1574	1015	

Arterial pressure usually rose during the injection, and slowly returned to the control level. The greatest rise was 40 mm. Hg. There was no correlation between the degree of rise and the gelatin fraction.

One dog (no. 14) succumbed following the injection of the high-molecular weight fraction. About 20 minutes following the injection, respiration became labored, arterial pressure began to fall. Death occurred 34 minutes after the injection, at which time blood was taken by cardiac puncture for the analyses.

reported. The plasma volume at this time was calculated by the "indirect" method and is probably too high. Post-mortem examination revealed mild diffuse late bronchopneumonia. There was much atelectasis and a number of alveoli and bronchioles contained eosin-staining fluid. Although the dog had bronchopneumonia, his appearance and control observations were normal, and his death was probably the result of the injection.

We are indebted to Miss R. Shore for the Kjeldahl determinations.

#### SUMMARY

From a bone gelatin average molecular weight 50,000 fractions were prepared with average molecular weights of 29,000, 36,000 and 69,000 by alcoholic precipitation. Solutions of these gelatines isoblemotic with dog blood plasma were injected intravenously in normal anesthetized dogs in a volume of 25 cc per kilogram, or an average of 63% of the control plasma volume. Retention of gelatin and of fluid in the circulation over a 4-hour period varied directly but not proportionally with the molecular weight. Retention does not increase markedly with increase in molecular weight above 50,000. By extrapolation, a fraction of this gelatin with average molecular weight of 25,000 or less can be expected to show little retention.

The fraction of injected gelatin excreted in the urine in 4 hours varies inversely with the molecular weight.

No reduction in total circulating plasma protein followed the injection of gelatin except in one experiment with the high molecular weight fraction.

None of the gelatin fractions reduced the amount of total circulating hemoglobin. All of the gelatin fractions caused some shrinkage of erythrocytes.

Sedimentation of erythrocytes was increased by all gelatin fractions most extensively by the high-molecular weight fraction.

One animal succumbed following the injection of high molecular weight gelatin.

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# ACUTE TOXICITY OF THIOUREA TO RATS IN RELATION TO AGE, DIET, STRAIN AND SPECIES VARIATION<sup>1</sup>

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It is only in the last few years that evidence indicating a high acute toxicity for thiourea has been published. Houben (1) surveying the literature in 1930 reported lethal doses of 10 grams per kgm. for frogs and 4 grams per kgm. for guinea pigs. He stated that 2 grams per kgm., intravenously was not fatal to dogs while 2 grams subcutaneously or intravenously caused only slight disturbance to rats. Pohl (2) in 1904 worked with dogs, cats and guinea pigs, and reported that thiourea was "completely non toxic." Binet (3) killed a 'young rat' (weight 120 grams) by injecting thiourea, but the dose he gave amounted to 5 grams per kgm. Flinn and Geary (4) found that 10 grams per kgm. killed 50 per cent of a group of rabbits (when given by stomach tube in aqueous solution) while 11 grams was required to kill 100 per cent. These authors stated that subcutaneous injections of the same doses (per kgm. body weight) into rats showed parallel results.

Hartzell (5-6), while studying chronic thiourea poisoning with rats fed 27.5 mgm. per kgm. daily to 30 rats over a period of 53 weeks, all survived with normal growth curves. On the other hand he found that the injection of 75 mgm. into the tail vein of a rat (weight not stated) killed it in less than 48 hours.

More recently Astwood and his co-workers reported (7-8) that feeding adult rats doses of thiourea up to 2 grams per day was not lethal over a ten day period.<sup>2</sup> Opposed to this was the observation of MacKenzie and MacKenzie (9) that of 24 adult rats given access to a stock diet containing 1 per cent thiourea, 22 died over night. These authors found, however, that weanling and immature rats survived doses up to 50 times as much (on a weight basis) as would kill an adult rat.

It had been observed in this laboratory that thiourea was of approximately the same high toxicity to rats as we had reported for the related compound phenyl thiourea (10). Both drugs produced symptoms of pulmonary edema and pleural effusion (as noted by MacKenzie and MacKenzie (9) and Binet (3) for thiourea) which are probably typical for all mono-substituted thiourea derivatives.

Ruling out the effect of age as a factor in the susceptibility of rats to thiourea

<sup>1</sup> The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

<sup>2</sup> Dr. Astwood informs us (private communication) that possibly the dose was 200 mgm. per day instead of 2 grams. This lower figure would be more consistent with our results (vide infra) although of course only the initial dose is significant for our purpose since large tolerances to thiourea are rather rapidly acquired by rats.

poisoning, there remain two main variables which might underlie divergent findings such as mentioned above. One is the strain or species of rat used, and the other is diet. This paper records work on strain and species variation in response to acute thiourea poisoning, together with some preliminary observations on the effect of diet and an experiment confirming the high resistance shown by young rats.

**EXPERIMENTAL DETAILS** We had at our disposal four kinds of rats, three strains of Norway rats (*Rattus norvegicus*) and one of Alexandrine rats (*R. rattus alexandrinus*).

1 Rats from our colony (herein referred to as "Hopkins"), most of whose forebears were albinos purchased 23 years ago from the Wistar Institute. Around 1928 several dozen pigmented rats from Dr E. V. McCollum's colony in the Johns Hopkins School of Hygiene were added; these rats had been inbred since 1917 and came originally from three sources, namely albinos from the Wistar Institute, "yellow" rats from the Bussey Institute at Harvard, and black pied rats from an animal fancier in Wisconsin. Our stock now consists of black and black hooded, tan and tan hooded and albino rats in about equal quantities.

2 Rats from Dr E. B. Astwood's colony in Harvard, the same as used in his studies (7) (8) (herein referred to as "Harvard" rats). This stock was obtained from Dr R. O. Greep in 1939 and stemmed from a colony of Long-Evans rats maintained at the Squibb Institute for Medical Research. Most of these rats were brown, resembling the wild Norway in appearance, while the rest were brown hooded, black or albino.

3 Wild Norway rats trapped in the streets and alleys of Baltimore by the Baltimore City Rat Control office. These were uniformly brown with tan or grey bellies.

4 Wild Alexandrine rats. Some were trapped in an office building in Baltimore and the rest were shipped to us from Gainesville, Florida, where they were trapped by Mr H. J. Spencer of the Fish and Wildlife Service. These were smaller rats with typically larger ears and longer tails than Norways; in color they were brown or black with grey, white or occasionally yellow bellies.

To determine the acute toxicity of thiourea<sup>3</sup>, the drug was administered simultaneously to groups of adult rats of the four different kinds, by intraperitoneal injection in olive oil suspension<sup>4</sup>. The results are therefore strictly comparable among each other, although the use of another medium can lead to different values for the median lethal dose, due probably to a change in rate of absorption (for example, compare values for adult "Hopkins" rats in table 1—olive oil—and table 3—aqueous solution).

To handle the wild rats without the aid of drugs or anaesthetics use was made of the Emlen sock (11), a slender cone of spaced wires terminating at the smaller

<sup>3</sup>The product used was Merck's Thiourea "pure."

<sup>4</sup>A suspension was used rather than an aqueous solution due to the large dosage range covered (1.0 to 4000 mgm per kgm body weight). To give a gram or more in solution would necessitate the administration of more than 12 cc. of fluid, which was considered undesirable.

end in a metal ring at the larger in a cuff of cloth or knitted fabric through which the rat enters. In this device rats can be readily weighed, held immobile for injection and then returned to their cages.

Hopkins' rats receiving fatal or near-fatal doses of thiourea exhibited sometimes in as little as 15 minutes symptoms of dyspnea, usually developing rales as the poisoning progressed. They sat hunched in their cages, eating and drinking little. Death occurred frequently but not always in convulsions. Harvard rats, on the other hand, seemed to outward appearance to be little affected by the drug, although in a series of these rats receiving 40-60 mgm per kgm and sacrificed at 1, 3, 7, 24, and 48 hours pleural effusion was found in every one, and edematous lungs in all but those killed at 48 hours. The wild Norways and Alexandrines appeared depressed but exhibited no dyspnea and usually died quietly, sometimes after considerable time in a comatose state.

Histological sections were made of most of the lungs and microscopic examination determined the existence or absence of edema. All diseased lungs were discarded at the same time.

At autopsy the characteristic findings after acute thiourea poisoning were pulmonary edema and pleural effusion. As an indication of the severity with which the drug affected the various rats it was a simple matter to measure these two effects quantitatively. The pleural fluid was drawn up into a graduated syringe and read in cubic centimeters, while the increase in weight of the lungs provided as good a measure as any for edema fluid (according to Wood and Moe (12)). Accordingly the pleural effusion observed in all rats succumbing to the drug was recorded in cubic centimeters per 100 grams of body weight. Likewise in each case the percentage lung weight was compared to a figure for the normal percentage lung weight.

By adding to the figure for average pleural effusion the average increase in lung weight (density of edema fluid assumed to be 1 which is probably sufficiently accurate for our purpose) a value for the total exudate was obtained which furnished a basis for comparison of the severity of the toxic effects in each group of rats.

**EFFECT OF STRAIN AND SPECIES VARIATION, AND CHANGES DUE TO DIET.** The results are given in tables 1 and 2. The LD 50's and their standard errors were estimated by the method of Litchfield and Fertig (13). It will be seen that a large variation in susceptibility existed between the three strains of Norway rats even when all were fed the same diet, whereas the difference in response between the two species of wild rats (wild Norways and Alexandrines) was not appreciable. Maintaining the wild Norways on our stock diet for a period of three to four weeks did not significantly lower the LD 50 from the value for those rats used right off the streets i.e. after being in the laboratory during a recuperative period of not more than 4 days. For Harvard rats however the change from Fox Chow to our stock diet for one month or more produced an almost fifteen fold increase in susceptibility.

The quantitative variation found in toxic effects is shown in table 2 which summarizes the data for survival time, pulmonary edema and pleural effusion pro-

duced in those rats of table 1 which died as a result of the poisoning. The total exudate in "Hopkins" and "Harvard" rats came out about the same, even for the "Harvard" rats fed Fox Chow, (which had so much greater resistance). It was slightly greater in the wild Norways fed our stock diet than in those from the street, while there was probably none in the Alexandrines (note normal lung weight is here greater than poisoned lung weight, only 3 of the 18 Alexandrines were found to have any pleural effusion at all).

TABLE 1

*Acute toxicity of thiourea to adult rats*

(administered intraperitoneally in olive oil suspension, no sex difference observed, about equal numbers of males and females in each group)

DIET AND NO. RATS USED	KIND OF RATS	BODY WEIGHT (AVERAGE AND RANGE)	TIME ON DIET	LD 50 $\pm$ S.E.	RATIO OF LD 50 S
				mgm per kgm	
Stock diet *		grams			
32	"Hopkins"	260 (172-402)	Since weaning	40 $\pm$ 0.2	1
18	"Harvard"	350 (209-482)	1-6 months	44 $\pm$ 13	11
18	Wild Norway	286 (164-504)	3-4 weeks	1340 $\pm$ 230	335
24	Alexandrine	140 (66-293)	2-4 months	1220 $\pm$ 230	305
Purina fox chow†					
10	"Harvard"	303 (234-396)	Since weaning	640 $\pm$ 191	160
Normal forage for city rats (4 days yellow corn meal after trapping)					
22	Wild Norway	346 (194-438)	Since weaning	1830 $\pm$ 135	458

\* Graham flour 72.5, crude casein 10.0, skim milk powder 10.0, butter 5.0, calcium carbonate 1.5, sodium chloride 1.0. This mixture ad lib., supplemented once weekly with lettuce.

† Guaranteed Analysis. Crude protein not less than 20%, crude fat not less than 3%, crude fibre not more than 6%, nitrogen free extract not less than 45%. Ingredients: wheat germ, dried skimmed milk, animal liver meal, brewers' dried yeast, barley malt, fish meal, meat meal, alfalfa meal, corn grits, soy bean oil meal, cereal feed (from corn and wheat), molasses, dried beet pulp, riboflavin supplement, vitamin A and D feeding oils, 1% steamed bone meal, 1% iodized salt.

**EFFECT OF AGE** To determine the effect of age on susceptibility thiourea was administered to 33 of our own rats ("Hopkins") ranging from 15 to 62 days old. Doses up to 800 mgm per kgm were administered by intraperitoneal injection, mostly in aqueous solution (because the size of some of the rats made it necessary to use a smaller needle than that required to inject olive oil suspensions). The experiment is summarized in table 3. It will be seen that whereas adult rats are likely to die following doses of 1.5 mgm per kgm or higher, suckling rats could survive 800 mgm per kgm without harm (Group C). 200 mgm per kgm was somewhat toxic but not fatal to 29 day old rats (Group E) as evidenced by a weight gain in 4 days of 10 grams against 15 for the rats in Group D receiving half

as much drug. Three of the 37 day old rats died following doses of 400 and 800 mgm. per kgm. (these were the *only* fatalities in the whole experiment), while all the 47 day old rats receiving 100 and 200 mgm. per kgm. lived although those in Group I exhibited an average weight loss of 15 grams in 4 days. Group K shows that even at two months of age rats could survive 400 mgm. per kgm. or more than two hundred times the dose fatal to adults.

This particular experiment was not continued above two months of age but among the 16 adults also receiving thiourea in aqueous solution were rats as

TABLE 2  
*Toxic symptoms produced by acute thiourea poisoning*  
(data given for those rats of Table I which died)

NO. DYING	KIND OF RATS	SUB-SURVIVAL TIME	PERCENTAGE PLEURAL EFFUSION (AVERAGE AND RANGE)		PERCENTAGE LUNG WEIGHT (AVERAGE AND RANGE)	NORMAL PERCENTAGE LUNG WEIGHT (AVERAGE AND RANGE)	TOTAL EX-DATE
			hours	cc. %			
Stock diet:							
22	'Hopkins	1-40	1.9	(0.1-3.6)	1.81 (0.85-3.70)	0.85 (0.56-1.16)	2.6
13	'Harvard'	1.3-70	1.6	(0.4-4.9)	1.56 (0.61-2.04)	[0.86]	[2.8]
10	Wild Norway	3-72	0.5	(0-1.6)	1.03 (0.72-1.46)	0.87 (0.85-1.37)	0.7
18	Alexandrine	3-72	0.03	(0-0.2)	0.55 (0.37-0.70)	0.78 (0.56-1.19)	?
Purina fox chow							
3	Harvard	13-60	2.7	(2.0-3.8)	1.16 (1.05-1.28)	[0.86]	[3.0]
Normal for age of wild city rats							
15	Wild Norway	17-40	0.2	(0-0.7)	0.82 (0.51-1.28)	0.87 (0.85-1.37)	0.25

Determined by weighing the lungs of healthy rats killed by breaking the cervical spine with pliers. 35 Hopkins, 46 Wild Norway and 27 Alexandrines were used. 'Harvard' rats lungs assumed to be intermediate in weight between 'Hopkins' and Wild Norways.

† Calculated as average pleural effusion plus average percentage lung weight less over age normal lung weight.

young as 3 months of age all of which exhibited the high susceptibility typical of adults every rat in this lot receiving 1.5 mgm. per kgm. or more died. It is at three months of age that the rats of our colony are considered sexually mature therefore it seems likely that the great drop in resistance occurs at the same time as the onset of puberty.

The rats in Groups A and B were from the same litter and were returned to the mother after injection. There was some leakage of the dose in Group A which received an olive oil suspension through a #19 needle (all subsequent injections

were made in aqueous solution with a #27 needle) The mother rat must have licked her young, because next morning she was found dead with typical symptoms of thiourea poisoning while all six young were alert and healthy. That the mother did not obtain the drug from the urine of her young was indicated in Group C these rats were separated from their mother for 30 minutes after injection and washed off and dried before being returned to the nest. This mother showed no evidence of poisoning, the same was true of the foster mother given to the 6 rats of Groups A and B.

**DISCUSSION** That so wide a variation in response to thiourea should exist between wild and domesticated rats, or rats of different species, is not so surprising, but to find rats from one colony of domesticated Norways 160 times as sus-

TABLE 3

*Influence of age on acute toxicity of thiourea to "Hopkins" rats*

Administered intraperitoneally in aqueous solution (except for Group A) LD 50 to adult "Hopkins" rats under same conditions =  $1.25 \pm 0.25$  mgm per kgm 16 rats used, weighing from 188 to 360 grams

GROUP*	AGE	DOSE	AVERAGE	NOTES
			WEIGHT OF RATS	
	days	mg per kgm	grams	
A	15	100	23	Survived
B	15	200	23	Survived
C	17	800	27	Survived
D	29	100	52	Survived av wt gain after 4 da = 15 grams
E	29	200	54	Survived av wt gain after 4 da = 10 grams
F	37	400	92	2 died in 3.5 hours with 4.3 and 3.5 cc of pleural effusion respectively, other survived
G	37	800	86	1 died in 3.5 hours with 3.4 cc of pleural effusion, other two survived
H	47	100	123	Survived av wt gain after 4 da = 7 grams
I	47	200	119	Survived av wt loss after 4 da = 15 grams
J	62	50	140	Survived
K	62	400	142	Survived

\* Three rats in each group

ceptable as those from another is remarkable. If adult rats can differ so much among themselves, it seems indicated that even greater caution should be used in applying the results obtained with rats to human beings. The standardization of diet for stock laboratory rats seems also indicated, since the change from one presumably complete diet to another in the case of the "Harvard" rats caused such a large shift in toxicity.

It may be that had the wild Norways been maintained longer on our stock diet they too would have exhibited a definitely lowered resistance. On the other hand a longer period in captivity would have brought in other factors, namely, the extensive adaptations involved in domestication. The wild Norways used in these studies were bold, fierce animals, with cunning and resourcefulness which

made the handling of each one an adventure. The Alexandrines were usually more timid but extremely agile while the two domesticated strains ('Hopkins' and 'Harvard') were very gentle offering no impediments to bare hand manipulation.

It is probable that the wild rats have a considerably higher basal metabolic rate than the domesticated ones. Anatomically, wild Norways have been found to differ from our domestic stock in having adrenal glands averaging three to five times heavier (14 c.f. also Donaldson 15), and most of the wild Norway rats autopsied in this laboratory had no retro-peritoneal fat in contrast to the large amounts of fat found in this region in our domestic rats.

Studies on the metabolism of thiourea, in man by Medes (16) and in rabbits by Blood and Lewis (17) indicate that the drug is excreted in the urine within 48 hours probably in unchanged form,<sup>4</sup> with positive tests for thiourea being obtained in urine collected 1 hour after administration. In agreement with this, we have observed that laboratory rats surviving 48 hours only occasionally succumb later, the large majority dying within 28 hours.

When given in sub-lethal doses over a period of time, thiourea has been found (7, 18) to produce chronic symptoms of lowered basal metabolic rate and thyroid hyperplasia together with the appearance of thyroidectomy cells in the pituitary gland. When, however, the poisoning is acute and the animal dies in a few hours there is no time for such symptoms to appear the only changes observed are those resulting from increased permeability of the pulmonary blood vessels and leading to pulmonary edema and pleural effusion.

We might postulate, therefore, that the greater resistance to thiourea of one strain of rat as opposed to another is due either to a difference in blood vessel permeability or to superior lymph drainage. There is however, still the question of diet to be completely investigated perhaps a change in the proportions of the various ingredients or the presence or absence of some as yet unidentified factor may make the difference. In this connection it is interesting to consider the observations of Hoskins Bloxham and Van Ess (19) who found thiourea to be lethal to flesh fly larvae as it is also to clothes moths but relatively non-toxic to leaf eating insects.

An explanation on the basis of increased protein in the diet may cover the strain variation, but it does not seem very likely that it would also explain the age variation in a given strain. Why such a large age variation should exist we have as yet no definite idea.

#### SUMMARY

1. In acute thiourea poisoning the median lethal dose, when administered under identical conditions varied greatly from one kind of rat to another. It was 4 milligrams per kilogram for tame Norway rats from our colony 44 for tame

<sup>4</sup> Pohl (2) stated that a very small fraction of the thiourea administered was converted to either methyl or ethyl sulfide detectable in the expired air by a garlic-like smell. We have observed this smell on the breath of one rat chronically poisoned with thiourea, and understand it has also been detected in patients receiving thiourea as medication.

Norway rats from Dr E B Astwood's colony at Harvard, and more than 1200 for wild Norway and wild Alexandrine rats, when all were being fed our stock laboratory diet

2 When Dr Astwood's rats were raised and maintained on Purina Fox Chow they showed greater resistance to thiourea the median lethal dose became 640 milligrams per kilogram compared to 44. Little difference was observed, however, between wild Norway rats which had been eating their normal forage outdoors and those fed our stock diet three or four weeks

3 Dr Astwood's rats and our own exhibited pulmonary edema and pleural effusion as the main toxic symptoms. The wild Alexandrine rats showed little or none of either symptom. The amount of both of these effects was slightly increased in wild Norway rats by feeding them our stock diet

4 Suckling rats and weanling rats up to 2 months of age from our own colony withstood doses of thiourea between 200 and 400 times as high as killed adults of the same strain under identical conditions

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# THE PHARMACOLOGICAL BEHAVIOR OF SOME DERIVATIVES OF SULFADIAZINE

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In investigating sulfonamides as chemotherapeutic agents in non-bacterial diseases we had occasion to examine the pharmacological behavior of a number of derivatives of sulfadiazine.<sup>3</sup> Since the methods were kept as uniform as possible, we believe the data to have value in illustrating the many unpredictable changes which occur in absorption, persistence in the blood, degree of acetylation, tendency to combine with plasma albumin and solubility when substituents are introduced into one or more of positions 4, 5 and 6 of the pyrimidine ring. These data are the subject of this report. No drug of the series proved to be superior to sulfamerazine as a chemotherapeutic agent in experimental bacterial diseases when all the factors just mentioned were taken into account.<sup>4</sup>

**Solubility determinations.** The solubility of both the free acids and their N<sup>4</sup>-acetyl derivatives in 0.05 M phosphate buffer at pH 6.6 and 37°C was determined. The amount of drug present was measured by the method of Bratton and Marshall (1). Duplicate tubes containing an excess of drug in suspension were always used. In one tube a saturated solution of the drug at 45°C was made; in the other saturation was completed at room temperature; both tubes were then rocked continuously at 37°C until the concentration of drug in each was the same. Thus equilibrium was established from both the supersaturated and the undersaturated states. Despite a number of experiments there were two instances [free acid of drug 14 and acetyl body of drug 21 (table 1)] in which the agreement between the two tubes was unsatisfactory.

**Measurement of protein binding.** The sodium salt of the drug being examined was dissolved (a) in the solution of van Dyke and Hastings (2) and (b) in the same solution containing 3 per cent human plasma albumin. The only protein contaminating the albumin were globulins which represented less than 1 per cent of the total protein. Identical concentrations of drug equivalent to 10 mg per cent of sulfadiazine were always present initially in each solution. Both solutions contained phenol red as an indicator (0.00075 per cent) and were equilibrated with a mixture of 5 per cent carbon dioxide and 95 per cent oxygen until the pH was 7.35-7.45. Seven ml of solution (b) were rapidly pipetted into a snugly tied length of "Viscose" sausage casing which also contained a marble nearly of the diameter of the casing. The sausage casing was immersed in 15 ml of solution (a) in a glass tube through an opening of one end of which the CO<sub>2</sub>-O<sub>2</sub> mixture was bubbled for 5 minutes longer to ensure a proper tension of CO<sub>2</sub> and the maintenance of a pH of about

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<sup>2</sup> All the new drugs of this series were synthesized in the Division of Medicinal Chemistry of this Institute under the direction of Mr. W. A. Lott. Chemical aspects of this work will be published elsewhere.

<sup>3</sup> The chemotherapeutic investigation of these drugs in bacterial infections was made under the direction of Dr. G. W. Rake in the Division of Microbiology and will be reported elsewhere.

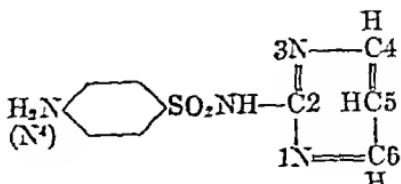
740 The tube was then tightly stoppered and rocked without interruption at 37°C. The marble gently stirred the protein solution continuously. Equilibrium was found always to have been established after 17 hours. At the end of equilibration, after solution (a) had been shown to be protein free, sulfonamide was determined in both solutions at a dilution of 1 to 200. The per cent sulfonamide bound is expressed in terms of water present in each solution, solution (a) contained 99 per cent water and (b) contained about 96 per cent water. In the actual calculation the difference between the concentration of sulfonamide in the water of the solvent containing protein (B) and in the water of the external fluid (A) divided by B and multiplied by 100 represents the "percent sulfonamide bound". At least duplicate determinations were always made and the values given in table 2 are believed to be accurate to  $\pm 4$  per cent (e.g. drug 1 could be 28-36 per cent bound) however, the usual agreement among repeated experiment was  $\pm 2-3$  per cent sulfonamide bound.

*Metabolism studies* All determinations of free or acetylated drug were made by the method of Bratton and Marshall (1) with slight modifications. The photo-electric colorimeter of Rosenfeld (3) as he has later modified it was used and its sensitivity was great enough so that 0.02-0.04 ml of mouse tail-blood was sufficient for a determination. When ever possible, the final dilution of blood or plasma, after the addition of trichloroacetic acid, was 1-200, lower dilutions had to be employed with low concentrations of drug. The drugs were determined in the whole blood of mice and in the plasma of monkeys. Plasma was preferred since the penetration of these heterocyclic sulfonamides into erythrocytes is variable and much less than that of sulfanilamide.

The desirable chemotherapeutic and metabolic characteristics of sulfamerazine led us to adopt this drug as a standard for comparison with the others. All the drugs were administered by stomach tube as aqueous solutions of sodium salts in a dose of 0.15 millimols per kilogram body weight (e.g. 43 mg sodium sulfamerazine dissolved in 25 ml water per kg mouse or in 5 ml water per kg monkey). A number of control experiments indicated that the peak blood or plasma level in both mice and monkeys occurred 2 hours after administration provided that food was withheld before the experiment. As a result of control experiments not reported here in detail, the persistence of these drugs in the mouse was determined 24 hours after administration.

The peak concentration of drug in monkey plasma appeared 2 hours after administration. Repeated determinations at 2, 4, 6, 10 and 24 hours demonstrated that the 10 hour plasma levels furnished a satisfactory measure of the persistence of free drug in the monkey and that 6-hour plasma levels appeared to be reliable to determine the degree of acetylation.

**RESULTS** *Solubility studies* Table 1 lists the structures of the various sulfapyrimidines investigated as well as the solubilities of the free acids and their N<sup>4</sup>-acetyl derivatives at pH 6.5 and 37°C. These compounds may be tabulated in a variety of orders, the order adopted, which may not conform to what many chemists would have chosen, is believed to be useful for comparing related compounds. All concentrations are expressed in terms of free sulfadiazine and were determined at least twice when, after repeated tests, the concentration of drug present was the same both in suspensions initially undersaturated (room temperature) and in those initially supersaturated (45°C). The formula of sulfadiazine and the system of numbering are



The effect of methyl substituents on solubility has been studied by others comparing sulfadiazine, 1, sulfamerazine, 2, and sulfamethazine, 3. We have studied, in addition to these the 4-5-6 methyl derivative 4 which is the least soluble of the four, its acetyl derivative has a particularly low solubility. Unfortunately the 5-methyl derivative could not be made. However, the insertion of a methyl ethyl methoxyethyl or ethoxyethyl group in the 5 position, when positions 4 and 6 were occupied by other aliphatic radicals led to a reduction of the solubility of both free acid and  $N^4$  acetyl derivative although there was no apparent consistency in the extent of diminution of solubility of free acid as compared with acetylated compound (cf. 3 and 4, 6 and 7, 19 and 20, 27 and 28, 6 and 30 and 6 and 17 in table 1). It appears likely that the aliphatic groups in positions 4 and 6 can also be important in determining the influence of a substituent in position 5 on solubility since a 4-R, 5-methoxy 6-R' compound could be more soluble (cf. 34 and 35) or less soluble (cf. 36 and 38) than the unsubstituted 4-R, 6-R' compounds; however, the  $N^4$  acetyl derivatives in both instances were less soluble after the addition of a 5-methoxy group.

If position 4 was occupied by an aliphatic group and a methyl group was added in position 6, the solubility of free acid and acetyl body was nearly always increased (the acetyl body of compound 9 is an exception, cf. 2 and 3, 5 and 6, 8 and 9, 10 and 11, 13 and 14, 18 and 19, and 23 and 24.) Free and acetylated compounds might be more soluble (cf. 2 and 3, 18 and 21 free acid only, and 18 and 33) or less soluble (cf. 5 and 27 and 8 and 31) when the same aliphatic group was in the 4,6 positions instead of in the 4 position only. The free acids of compounds of the type 4-R, 6-R' were consistently more soluble than the simpler 4-R compounds (cf. 23 and 25, 23 and 26, 13 and 34, and 13 and 36). Ethyl mercapto compounds (40 and 41) were remarkably insoluble.

Since the majority of these compounds are acetylated in the course of metabolism, the relative solubilities of free acid and acetyl derivative are of considerable interest. In 3 instances both had the same solubility, in 15 comparisons the free acid was the more soluble whereas in the other 20, this was true of the acetyl body.

How solubility is related to the dissociation of these compounds as acids is unknown. The  $pK_a$  values of a few are known (4) and their solubilities as determined by us were

DRUG	pKa(4)	SOLUBILITY AS $\mu$ M/L AT pH 6.5 AT 37°C.
Sulfadiazine 1	6.48	1.20
Sulfamerazine 2	7.06	1.64
Sulfathiazole	7.12	5.20
Sulfamethazine 3	7.37	3.00

It is evident that there is no simple correlation of solubility with the  $pK_a$ . It is also evident that it would be desirable to determine the solubility of all compounds at various hydrogen ion concentrations at which the dissociation of the compounds would be so low as to make the measured solubility that of the un-

dissociated molecules, or so high as to make the measured solubility that of the ions

*Measurements of protein binding* The methods of making these measurements and of calculation have already been described. The data, which are limited to a study of the non-acetylated acids only, are presented in table 2. Three of the non-acetylated acids, 10, 28 and 41, were so insoluble even at pH 7.4 that observations could not be completed. About 0.01 mM of sulfonamide was bound by one gram of albumin when 50 per cent of the sulfonamide originally in solution was bound under the conditions of these experiments.

By comparing tables 1 and 2, the reader can convince himself that there is no correlation between solubility at pH 6.5 and the apparent binding of these sulfonamides by human plasma albumin at pH 7.4. The addition of one or more methyl substituents seemed to be accompanied by increased protein-binding (cf. 1 with 2, 3 and 4), but this was not necessarily the case if the 4 position was already occupied by another group (cf. 5, 6 and 7, 8 and 9, 13 and 14, and others). A comparison of drugs 5 and 27, and 8 and 31 suggested that the same substituents in the 4,6 position led to a greater binding by protein than would have occurred if only the 4 position was occupied, however, this was not true of all aliphatic groups (cf. 2 and 3, and 13 and 33). To what extent specific groups alter the degree to which derivatives of sulfadiazine are bound by plasma albumin is difficult to decide from the available data. However, it is clear that the replacement of even one hydrogen atom by an aliphatic radical in position 4 of the pyrimidine ring of sulfadiazine will probably lead to an increased binding of the sulfonamide to albumin. It is likely that specific groups rather than number of substituents greatly influence further changes, if any, as the hydrogen atom of positions 5 or 6 or both are also replaced (e.g. compare 2 and 3 with 5 and 27, and 8 and 31).

So far as studies of identical compounds are concerned and the differences among methods are taken into account, the results are in general agreement with those of other authors (5, 6, 7). Gilligan (7) concluded that acid ionization at pH 7.4 could not be correlated with degree of protein-binding. The pKa values of most of the drugs used have not been determined but it does not seem likely that these could have been correlated with the per cent of sulfonamide bound to albumin.

*Metabolic studies* The metabolism of the compounds listed in table 1 was investigated in mice and monkeys. The results were obtained from groups of at least 5 mice or 2 monkeys unless the data were variable and demanded administration of drug to more animals. All the drugs were given as solutions of sodium salts by stomach tube in a dose of 0.15 mM per kilogram body weight. For purposes of comparison, sodium sulfamerazine was chosen as the standard. In table 3, it is given as 100 and this value as obtained from 85 mice and expressed as blood sulfamerazine was 9.5 mg per cent after 2 hours and 1.7 mg per cent after 24 hours. In monkeys the same dose was used but the plasma drug-levels were determined after 2, 4, 6, 10, 24 and, if necessary, 48 and 72 hours. In 9 experiments in monkeys, the peak plasma sulfamerazine reached after 2 hours

TABLE 1

Solubility of sulfapyrimidines and their 4-acetyl derivatives suspended in 0.05 M phosphate buffer pH 6.5 at 37°C

DRUG NO	POSITION OF SUBSTITUENT IN PYRIMIDINE RING			FREK ACID	ACT TTL DOSI- TIVE*
	4	5	6		
1	-H	-H	-H	30	80
2	-CH <sub>3</sub>	-H	-H	41	50
3	-CH <sub>3</sub>	-H	-CH <sub>3</sub>	75	75
4	-CH <sub>3</sub>	-CH <sub>3</sub>	-CH <sub>3</sub>	10	1
5	-H	-H	-OCH <sub>3</sub>	46	33
6	-CH <sub>3</sub>	-H	-OCH <sub>3</sub>	165	45
7	-CH <sub>3</sub>	-CH <sub>3</sub>	-OCH <sub>3</sub>	12	10
8	-H	-H	-OCH <sub>2</sub> CH <sub>3</sub>	9	14
9	-CH <sub>3</sub>	-H	-OCH <sub>2</sub> CH <sub>3</sub>	220	12
10	-H	-H	-OCH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	0.6	4
11	-CH <sub>3</sub>	-H	-OCH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	60	14
12	-CH <sub>3</sub>	-H	-OC <sub>2</sub> H <sub>5</sub>	8	
13	-H	-H	-CH <sub>2</sub> OCH <sub>3</sub>	50	63
14	-CH <sub>3</sub>	-H	-CH <sub>2</sub> OCH <sub>3</sub>	50-75	94
15	-CH <sub>3</sub>	-H	-CH <sub>2</sub> OCH <sub>2</sub> CH <sub>3</sub>	26	35
16	-CH <sub>3</sub>	-CH <sub>2</sub> CH <sub>2</sub> OCH <sub>2</sub> CH <sub>3</sub>	-H	9	11
17	-CH <sub>3</sub>	-CH <sub>2</sub> CH <sub>2</sub> OCH <sub>2</sub> CH <sub>3</sub>	-OCH <sub>3</sub>	102	8
18	-H	-H	-OCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	27	40
19	-CH <sub>3</sub>	-H	-OCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	170	>150
20	-CH <sub>3</sub>	-CH <sub>3</sub>	-OCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	63	34
21	-OCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	-H	-OCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	100	33-40
22	-CH <sub>3</sub>	-H	-CH(OCH <sub>3</sub> ) <sub>2</sub>	51	59
23	-H	-H	-CH(OCH <sub>3</sub> CH <sub>3</sub> ) <sub>2</sub>	8	63
24	-CH <sub>3</sub>	-H	-CH(OCH <sub>3</sub> CH <sub>3</sub> ) <sub>2</sub>	33	
25	-OCH <sub>3</sub>	-H	-CH(OCH <sub>3</sub> CH <sub>3</sub> ) <sub>2</sub>	29	29
26	-CH <sub>2</sub> OCH <sub>3</sub>	-H	-CH(OCH <sub>3</sub> CH <sub>3</sub> ) <sub>2</sub>	82	
27	-H	-H	-OCH <sub>3</sub>	46	33
28	-OCH <sub>3</sub>	-H	-OCH <sub>3</sub>	20	12
29	-OCH <sub>3</sub>	-CH <sub>2</sub> CH <sub>3</sub>	-OCH <sub>3</sub>	0.2	1
30	-OCH <sub>3</sub>	-CH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	18	2
31	-CH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	-H	-OCH <sub>3</sub>	55	18
32	-H	-H	-OCH <sub>2</sub> CH <sub>3</sub>	9	14
33	-OCH <sub>2</sub> CH <sub>3</sub>	-H	-OCH <sub>2</sub> CH <sub>3</sub>	3	5
34	-H	-H	-H	31	8
35	-CH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	-H	-CH <sub>2</sub> OCH <sub>3</sub>	50	63
36	-H	-H	-CH <sub>2</sub> OCH <sub>3</sub>	108	150
37	-OCH <sub>2</sub> CH <sub>3</sub>	-H	-CH <sub>2</sub> OCH <sub>3</sub>	75	85
38	-OCH <sub>3</sub>	-H	-CH <sub>2</sub> OCH <sub>3</sub>	140	60
39	-OH	-H	-CH <sub>2</sub> OCH <sub>3</sub>	46	33
40	-H	-H	-CH <sub>2</sub> OCH <sub>3</sub>	110	100
41	-SCH <sub>2</sub> CH <sub>3</sub>	-H	-CH <sub>2</sub> OCH <sub>3</sub>	55	55
		-H	-CH <sub>2</sub> OCH <sub>3</sub>	90	6
		-H	-CH <sub>2</sub> OCH <sub>3</sub>	100	110
		-H	-SCH <sub>2</sub> CH <sub>3</sub>	1	3
		-H	-SCH <sub>2</sub> CH <sub>3</sub>	0.1	1

As mg. percent of sulfadiazine which would be equivalent to the measured solubilities of the compounds listed. These figures may be converted into mM/l by multiplying by 0.04

\* To facilitate comparisons in the case of this drug and others in the table, the usual rule of numbering substituents which would require that this compound be the 4-methoxy derivative is not followed.

was 17.6 mg per cent, after 10 hours, the plasma level of free drug was 4.4 mg per cent, after 6 hours, acetylation appeared to be at a maximum and represented 8 per cent of total sulfamerazine present (9.3 mg per cent in plasma). In both mice and monkeys, peak levels were obtained 2 hours after oral administration. However, mice consistently excreted the drugs more slowly and persistence was measured by the blood level after 24 hours, whereas reliable data

TABLE 2

*The extent to which the free acids of table 1 were bound by human plasma albumin*

DRUG NO	PER CENT* BOUND	DRUG NO	PER CENT BOUND
1	32	23	67
2	57	24	78
3	58	25	81
4	61	26	79
5	50	5	50
6	55	27	78
7	65	28	Insoluble
8	60	29	
9	55	30	56
10	Insoluble	8	60
11	67	31	88
12	92	32	66
13	66	13	66
14	62	33	64
15	64	34	77
16	75	35	79
17	58	5	50
18	45	36	67
19	43	37	64
20	45	38	78
21	57	39	82
22	68	40	77
		41	Insoluble

\* A = sulfonamide in 100 ml of protein free water external to the Viscose membrane

B = sulfonamide in 100 ml of water in the protein solution confined by the Viscose membrane

$$\text{Per cent bound} = \frac{100(B - A)}{B}$$

Before equilibration, A and B each contained 0.04 mM sulfonamide or 0.4 mM/l

in the monkey could not be secured this late and the 10-hour level seemed to furnish the best guide

It was found difficult to summarize the large amount of data gathered but the method employed in table 3 seemed as convenient as any. Sulfamerazine, always given a value of 100, was compared with all the other drugs. Drug 3 may be used as an example for readers who would prefer to ascertain exact values. The 2 and 24 hour blood levels of free drug in mice, with correction for difference in molecular weight, were 6.0 and 0.7 mg per cent, the 2 and 10 hour plasma levels in monkeys were 9.3 and 0.9 mg per cent, 25 per cent of the total

TABLE 3

The absorption, persistence and conjugation of sulfapyrimidines in terms of sulfamerazine (drug A)

Absorption and persistence are based upon free drug levels; conjugation on per cent of total drug acetylated

SERIAL NO.	MOSA (MUCOP)		MOSA (PLASMA)		
	Absorption (2 hr level)	Persistence (24 hr level)	Absorption (2 hr level)	Persistence (10 hr level)	Conjugation (8 hr level)
1	70	50	80	40	110
2	100	100†	100‡	100§	100
3	00	40	50	20	300
4	<30	0	40	10	120
5	70	40	40	20	40
6	110	200	30	<10	60
7	80	140	30	20	120
8	70	40	40	30	170
9	100	240	40	20	310
10	<30	<30	10	20	0
11	70	10	40	10	20
12	80	30	90	100	120
13	80	60	100	130	0
14	80	30	100	90	170
15	80	30	100	30	0
16	80	30	100	160	0
17	<30	<30	30	10	120
18	<30	<30	40	0	0
19	<30	<30	30	10	200
20	<30	<30	10	<10	300
21	<30	<30	50	120	<60
22	<30	<30	100	200	0
23	110	70	80	130	60
24	120	80	120	260	100
25	80	80	90	200	240
26	80	80	90	160	180
27	100	230	100	160	350
28	30	0	10	20	40
29	60	40	80	70	400
30	<30	0	40	<10	320
31	70	300	30	80	170
32	70	90	60	100	0
33	80	60	100	130	0
34	110	60	90	60	0
35	80	160	80	160	80
36	80	60	90	80	120
37	70	130	100	90	300
38	70	160	100	50	<80
39	80	40	110	190	120
40	80	<30	00	160	0
41	<30	0	10	30	0

0.8 mg. per cent free sulfamerazine in blood.

† 1.7 mg. per cent free sulfamerazine in blood

‡ 17.6 mg. per cent free sulfamerazine in plasma.

§ 4.4 mg. per cent free sulfamerazine in plasma.

|| 8 per cent of total sulfamerazine conjugated (0.8 mg. per cent total drug in plasma)

|| Determined at 2 hours; levels too low at 8 hours.

At 8 hours (not 6)

|| At 10 hours (not 6)

sulfamethazane (drug 3) in the plasma of monkeys was conjugated at 6 hours. Too much weight must not be given to the figures of table 3 since, for purposes of comparison, these are all referred to sulfamerazine as a standard.

In the mouse a particular drug may be absorbed better (e.g., 6, 7, 9 and 31) or more poorly (e.g. 16, 24, 25 and 39) than in the monkey. In the remaining experiments there was quite satisfactory agreement. The observations on persistence are not as directly comparable since the levels were measured after 24 hours in the mouse and after 10 hours in the monkey. The results in the two species disagreed often, persistence was sometimes greater in the mouse (e.g. 6, 7, 9, and 37) but more often was greater in the monkey (e.g. 12, 13, 14, 16, 21, 22, 24, 25, 26, 38 and 39). It seems probable that the data in monkeys have more significance with reference to the behavior of these drugs in man. The degree of conjugation in mice was not determined.

It must be recognized that the headings used in Table 3 are not to be taken too literally. If absorption were truly measured, the total drug excreted in the urine and by other routes would have to be determined. Persistence may in part depend upon the rate of absorption and could, of course, itself affect what is here loosely termed absorption. Absorption, as the term is used in table 3, does not depend upon solubility alone although drugs with solubilities of 6 mg per cent or less (table 1) were usually poorly absorbed (10, 28, 40 and 41 except 31). Persistence likewise did not depend upon solubility. Drugs in which aliphatic radicals had replaced hydrogen in the 4, 5 and 6 positions were often poorly absorbed and persisted poorly (4, 17, 20, 28 and 30) although there were exceptions (35 and 38). The table contains examples of the manner in which absorption and persistence were affected by specific groups (cf. 2, 13 or 23 with 5, 8, 10 or 18) when the aliphatic group was in the 4 position. A study of tables 1 and 3 indicates that the absorption and persistence of a compound might also be strikingly altered by the addition of a group to the 6 position which previously had had no substituent (e.g. 16 and 17 in the monkey). Absorption and persistence, at least in the monkey, could be improved or worsened if the same group was the substituent in the 4,6-positions instead of in the 4 position alone (e.g. cf. 2 and 3, and 5 and 27), apparently the substituting group determined what metabolic change, if any, occurred.

Conjugation, which is assumed to be acetylation of the free N<sup>4</sup> amino group, was studied only in the monkey. Like absorption and persistence, it varied greatly even among related compounds. An increase in conjugation was not considered to have taken place unless the proportion of drug conjugated was at least doubled, virtually no drug was conjugated in those cases in which a diminution was believed to have occurred. It did not depend upon the solubility of either the free acid or the N<sup>4</sup>-acetyl derivative. With the single exception of drug 39, in which one substituent was an hydroxyl group, all the compounds with substituents in the 4,5 and 6 positions were conjugated as much as or more than sulfamerazine. The conjugation of 4,6- substituted derivatives might be either greater than (2 and 3, 8 and 9, 13 and 14, 18 and 19,

and 23 and 25) or the same as (5 and 6, 23 and 24, 18 and 21, 5 and 27, and 13 and 33) 4-substituted derivatives, four of six di-substituted compounds in which one group was a methyl were more conjugated than the simpler 4 substituted drug. The 4,6-diethoxy derivative, 31 was conjugated more than sulfamerazine whereas there was a striking lack of conjugation of the 4,5 diethoxy derivative, 32. The monkey conjugated virtually none of the other two 4,5-substituted derivatives (16 and 37). It should again be mentioned that no conjugation of the 4-hydroxy 5-ethoxy 6-ethoxymethyl pyrimidine derivative 39 occurred in the monkey. Other compounds of which no conjugation was observed following administration to monkeys were drugs 13, 33, 15 and 22 (4 or 4,6 methoxymethyl, 4 methyl and 6 ethoxymethyl or 6 dimethoxymethyl derivatives). It appears that specific groups may markedly affect the degree of conjugation.

Persistence of these sulfonamides in the blood or plasma cannot be satisfactorily correlated with their binding by albumin. For example drug 12, of which a significantly greater proportion was bound by plasma albumin than of drug 2 persisted no better (monkey) or less well (mouse) than drug 2. Persistence less satisfactory than that of sulfamerazine was observed not only of all drugs of which 50 per cent or less were bound by albumin (e.g. 1, 5, 18, 19 and 20) but also of some in which the percentage of sulfonamide bound equaled that of sulfamerazine (e.g. 3, 33, 37). Drugs which persisted better than sulfamerazine (22, 24, 25, 38 and perhaps others) were bound to albumin to the extent of 70-80 per cent in comparison with about 60 per cent for sulfamerazine.

#### SUMMARY

1 The influence of aliphatic substituents in one or more of positions 4,5 and 6 of the pyrimidine ring of sulfapyrimidines on the solubility of the free and  $N^4$  acetyl-substituted compounds in 0.05 M phosphate buffer, pH 6.5 was studied at 37 C. In the majority the acetyl derivative was the more soluble whatever the relationship. It was often strikingly altered by the addition (or removal) of one or more non polar groups. In comparison with 4- or 4,6- substituted sulfapyrimidines, 4,5,6- derivatives were nearly always less soluble. If position 4 was occupied the addition of a methyl group to position 6 usually increased solubility. Other compounds of the type 4-R, 6-R' were more soluble than 4-R compounds whereas compounds 4-R, 6-R might or might not be more soluble than 4-R compounds. It is believed that the dissociation of a compound as an acid is not the only factor determining solubility.

2 The extent to which the various derivatives of sulfadiazine were bound by human plasma albumin was measured. The introduction of one or more aliphatic radicals in positions 4, 5 or 6 of the pyrimidine ring always led to an increased binding of sulfonamide. Specific aliphatic groups appeared to affect the degree of binding.

3 The absorption and persistence of these sulfonamides were studied in the mouse and monkey to the extent that conclusions can be reached from

drug-levels in blood or plasma. The degree of conjugation was also determined in the plasma of monkeys. There were frequent disagreements between the results in mice and those in monkeys.

Poor absorption usually was characteristic of drugs of low solubility but could also characterize much more soluble compounds. Specific aliphatic groups and their position appeared to be the principal factors determining absorption, persistence and magnitude of conjugation. The degree of binding to plasma albumin did not alone determine persistence.

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## A CHEMICAL EVALUATION OF DIGITALIS<sup>1</sup>

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Although nearly 50 years have passed since the first published work on the biological assay of digitalis appeared there is still active controversy among the proponents of the various biological methods available. This leads to the conclusion that none of the present methods is entirely satisfactory.

At the suggestion of the Chairman of the Committee of Revision of the U S Pharmacopoeia we undertook an examination of the chemical methods available for the assay of digitalis.

The first qualitative chemical test for digitalis glycosides was published by Homelle (1) in 1845. This test similar to most of the numerous tests developed since that time is a color reaction. The adaptation of these tests for a quantitative colorimetric procedure is not usually practicable, as these tests usually require relatively pure glycosides.

Attempts aimed at the quantitative isolation of the active glycosides, with subsequent gravimetric or chemical determination, have not proved very successful.

Of the colorimetric methods proposed it seemed to us that the Knudson and Dresbach (2) method offered the most promise for restudy. This method is based upon the Baljet (3) reaction in which a red-orange color is developed by the active glycosides in the presence of alkaline picrate solution.

More recent work (4) has shown that this color reaction is due to the active hydrogen atom of the unsaturated lactone group which is characteristic of the cardiac glycosides. Although other substances respond positively to the Baljet test it is fortunate that it is the lactone group in the glycoside molecule which gives the response because Jacobs (4) has shown that cardiac activity depends upon this structure.

In our experiments we employed the procedure of the Knudson Dresbach method modifying it by the use of the Electrophotometer to compare colors. The instrument used was a Fisher Electrophotometer. The green filter No 525 was employed in conjunction with the 23 cc absorption tubes. This combination was found to give in general readings near the most desirable portion of the scale. All readings were made on the logarithmic scale of the instrument. Individual readings could be completed in less than 15 seconds so that the course of the color development with the time could be followed very conveniently. The solution used as a blank consisted of equal volumes of distilled water and the alkaline picrate solution.

<sup>1</sup>The expense of the investigation was defrayed partially by a grant from the Board of Trustees of the United States Pharmacopoeial Convention.

Our attention was first directed to an examination of the color development in aqueous ouabain solutions prepared from the U S P Reference Standard material which contained respectively 20, 15, 10 and 5 mg per 100 cc of solution. Readings were made over a period of 90 minutes. The data obtained for each solution were transposed to graphical form by plotting the readings (logarithm of the transmission) against time. The four curves thus obtained were very similar and practically parallel to one another beyond the first 10 minute reading as shown in chart 1. Readings of the transmission at the same time interval for each curve were then plotted against the corresponding concentration of ouabain

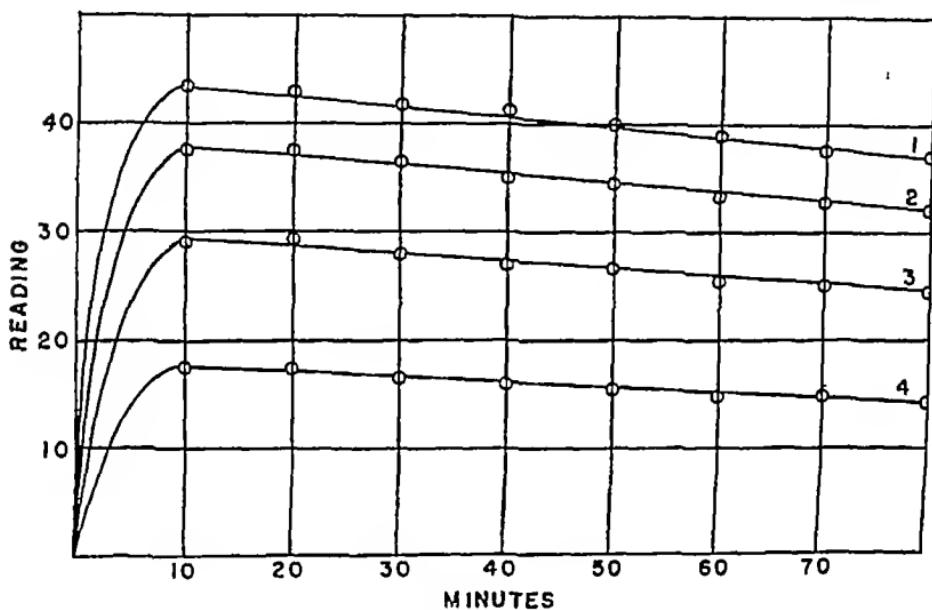


CHART 1

## OUABAIN SOLUTIONS

- 1 20 mg /100 cc
- 2 15 mg /100 cc
- 3 10 mg /100 cc
- 4 5 mg /100 cc

The locus of these points is not a straight line but shows considerable curvature indicating a definite divergence from Beer's law. The curvature is less pronounced in the lower concentration range.

The four curves reveal another interesting and important fact in that the maximum reading for each curve was obtained at the first ten minutes interval after which subsequent readings showed a consistent and significant decrease. In other words, the color developed in ouabain solutions in the concentration range of 5 to 20 mg per 100 cc begins to fade after 10 minutes and probably even before that time. Tinctures of digitalis, on the other hand, examined by the

same method developed a color with alkaline picrate solution which became more intense with respect to time (as shown in chart 2)

In addition, we examined solutions of dextrose and digitoxose in a concentration of 1 mg per cc over a period of several hours after treatment with the picric acid reagent. Confirming other published observations we found that these solutions give the characteristic color reaction which gradually increases in intensity with time. Dextrose is considerably more active than digitoxose. Compared with the behavior of solutions containing digitalis glycosides the effect of these sugars is not regarded as significant, as shown by the accompanying chart

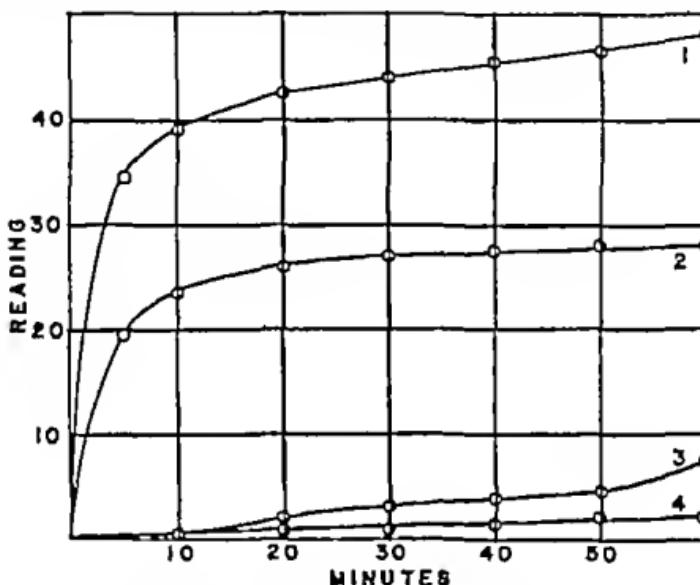


CHART 2

## DIGITALIS GLYCOSIDES AND SUGAR SOLUTIONS

1 and 2 Typical curves for U.S.P. Reference Standard tinctures in full and half strength respectively

3 Dextrose 1 mg /cc

4 Digitoxose 1 mg /cc.

CHART 2 An extensive series of time-transmission studies of tinctures of digitalis originating from a variety of specimens of the drug were made. The studies included tinctures of different but known strength and dilutions thereof. As a result of these observations we suggest the following method of assay employing the U.S.P. Reference Standard Tincture as the standard of comparison.

The method requires 5 cc of the unknown tincture and 5 cc of a tincture prepared from the U.S.P. Reference Standard Powder and also 5 cc of the latter after dilution with an equal volume of 71 per cent alcohol. The three tinctures are decolorized by the Knudsen Dresbach procedure in separate 25 cc. volumetric

flasks as follows. Approximately 15 cc of water is added to exactly 5 cc of tincture in the first flask followed by 2 cc of a freshly prepared 12.5 per cent solution of lead acetate. The contents of the flask are mixed, then diluted to the mark with water, mixed again and transferred to a filter. During the filtration (by gravity) from the first flask, the 2nd and 3rd flasks are carried through the same procedure. When 15-20 cc of the first filtrate have been collected, 12.5 cc are transferred to a 25 cc volumetric flask and 2 cc of a 4.7 per cent solution of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  is added. The contents of the flask are mixed, diluted to the mark with water, mixed again and then transferred to a filter. The other two lead acetate filtrates are treated in the same manner. Twelve and five tenths cc portions of each of these 3 filtrates are placed in suitable individual containers. The picric acid reagent is prepared freshly by mixing stock aqueous solutions of 10 per cent sodium hydroxide and 1 per cent picric acid in the proportion of 5 cc of the former to 95 cc of the latter. Twelve and five tenths cc of this reagent is mixed with 12.5 cc of water and transferred to the proper absorption cell in the photoelectric colorimeter. This solution serves as a blank. The same volume of the reagent is also added and immediately mixed with each of the 3 filtrates noting the time when each addition was made. The additions may be made conveniently 2 or 3 minutes apart which permits ample time for the adjustment of the instrument and reading of the transmission before the next solution is to be examined. The solutions are transferred to absorption cells and the transmission of each solution is measured exactly 20 minutes after the picric acid reagent has been added. The readings are made to the nearest 0.5 or whole division on the scale.

The percentage potency of the unknown tincture in terms of the U S P Reference Standard tincture is obtained from the equation

$$\text{Potency} = 50 \left( \frac{R_x - R_{100}}{R_{100} - R_{50}} \right) + 100$$

where the values of  $R_x$ ,  $R_{100}$  and  $R_{50}$  are respectively the instrument readings (logarithm of the transmission) corresponding to the unknown tincture, U S P Reference Standard tincture and U S P Reference Standard tincture in half strength. In table 1 is given a summary of 50 typical pairs of readings of the full and half strength U S P Reference Standard tincture observed during the course of this study. Substituting the appropriate average values in the foregoing equation we have

$$\text{Potency} = 50 \left( \frac{R_x - 42.5}{16.5} \right) + 100$$

In table 2 are listed a limited series of results obtained by this method on coded biologically assayed preparations supplied us by Dr Harry Gold. Sample No 1 is the 15 year old sample of tincture prepared by Dr James C. Munch. From this limited series it is apparent that the results obtained by the proposed method much more closely parallel those by the cat method than those obtained by the human method.

TABLE 1

Statistical analysis of fifty pairs of readings of full and half strength U.S.P. Reference Standard Tinctures

	AVERAGE	MEDIAN	MAX. VALUE	MIN. VALUE	STANDARD ERROR	STANDARD ERROR PER CENT
Full strength	42.5	42.5	45.0	39.5	0.17	0.40
Half strength	26.0	26.0	27.5	24.5	0.10	0.38
Difference	16.5	16.5	18.0	14.0	0.11	0.67

TABLE 2

SAMPLE	PER CENT POTENCY IN TERMS OF STANDARD		
	Chemical	Cat	Human
No. 1 Tincture	104	100	
	104		
	102		
No. 2 Powder	105	132	109
	106		
No. 3 Powder	117	115	
	115		
No. 4 Powder	118	109	
	126		
No. 5 Powder	113	105	
	113		
No. 6 Tincture	155	140	107
No. 7 Powder	152	160	94
	157		
No. 8 Powder	132	140	107
No. 9 Tablets	112	100	89.5
	109		
No. 10 Tablets	126	115	113
	119		
No. 11 Powder	132	132	115
	142		
No. 12 Powder	161	163	119.8
	165		
No. 13 Tincture	151	161	91.1
	154		

To study the value of this method in the hands of other workers Dr Harry Gold sent us 5 coded samples of digitalis powder assayed by the cat and human methods (5). We recoded these samples and sent them to six independent laboratories including Dr Gold's laboratory. The results of these collaborative studies are set forth in table 3.

DISCUSSION A chemical method for the assay of digitalis has been proposed dependent on the Baljet reaction and the Knudson-Dresbach procedure. Based

TABLE 3

Code designation of sample	CHEMICAL ASSAY							Identity of sample	BIOASSAY				
	Per cent potency in terms of standard								Cat		Human		
	Collaborators								S.E. per cent	Per cent potency in terms of standard	Potency in terms of standard	S.E. per cent	
	1	2	3	4	5	6	7		Unknown	Standard			
A = 5	131	119	108	121	131	126	133	124	2.7	Reference Standard XI	131	5.2	
					132		134				132		
							131						
B = 4	130	122	128	120	137	132	137	128	1.7	H.A. No 9	107	6.3	
					138		128						
							120						
C = 3	123	108	93	105	104	114	107	107	3.7	L No 5	102	4.7	
				107		107							
							108						
D = 2	96	102	94	102	110	94	105	100	2.2	Reference Standard XII	100		
					107		105						
							104						
E = 1	79	89	83			91	90	87	2.8	L-300 H 2895	105	3.1	
							91						

## Key to collaborators

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- 2 Dr Harry Gold, Department of Pharmacology, Cornell University Medical College
- 3 Dr C. I. Bliss, Department of Pharmacology, Yale University
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\* Assayed by Dr Harry Gold

upon the work of Jacobs, namely, that the active hydrogen of the unsaturated lactone group of the glycosides is necessary for cardiac activity, the Baljet reaction measures pharmacologic potency. In these studies we have shown that the old incrimination of the Knudson-Dresbach procedure, namely, that it was measuring reducing sugars, is not valid under the conditions of this assay.

Data are recorded to show that the agreement between assays obtained by this method and those obtained by the U. S. P. cat method is satisfactory, certainly within the necessity of agreement for this assay. The U. S. P. permits a 57

percentage S.E. for the official cat method in the hands of one operator in our hands seven values taken at random for the standard U.S.P. tincture showed a percentage S.E. of 1.2. From table 3 it will be observed that in different hands for the first experience with the test, although skilled in the manipulations of the chemist the average percentage S.E. was 2.6 and that obtained by the one skilled bioassayist by the cat method was 3.8. Dr C. I. Bliss (6) very succinctly states in his monumental mathematical review of the collaborative studies of the U.S.P. cat method, that this method will show a difference of 20 per cent between two tinctures, but not 15 per cent.

That time and expense are factors in this procedure goes without saying. Even skilled bioassayists require 4 to 7 hours to obtain a value by the cat method with the sacrifice of 7 or more cats. With the proposed method the expense is negligible the skill required is that of the average technician and the time 45 minutes.

#### CONCLUSION

1 A chemical method has been proposed to assay digitalis and its preparations that agrees well with the U.S.P. cat method. The Baljet reaction and the Knudsen-Dresbach decolorization procedure form the basis of the assay.

2 The unavailability of ouabain as a suitable standard of comparison has been shown. Reference Standard Digitalis Powder has been successfully employed as a standard.

3 The relative importance of the time factors in the development of the color for lactone grouping in the glycoide and the reducing sugars respectively has been emphasized.

4 Success in the quantitative evaluation of the color in this assay is dependent upon the use of a photoelectric colorimeter provided with a suitable filter.

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# STUDIES ON SHOCK INDUCED BY HEMORRHAGE

## IX. THE INHIBITION OF AMINO ACID OXIDATION IN SHOCK INDUCED BY HEMORRHAGE<sup>1</sup>

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### 1 INHIBITION OF AMINO ACID OXIDATION IN KIDNEY

It has been shown by Lurje (1) that in shock the concentration of amino acids in the blood is increased. This has been confirmed by Engel et al (2) who have shown that in eviscerate (liverless) rats subjected to hemorrhage, the increase in blood amino acids is much more rapid than in normal animals similarly treated. Such an accumulation of amino acids might be due to an increase in protein catabolism, or to inhibition of amino acid metabolism, or to both. In this laboratory it has been shown that certain metabolic disturbances which occur in dogs subjected to shock may be attributed to a breakdown of coenzymes. Destruction of the apoenzyme, or protein portion of the enzyme, has also been shown to occur under conditions of anoxia in *in vitro* experiments. The following series of experiments has been conducted to determine whether shock from hemorrhage does result in impairment of the ability to oxidize certain amino acids.

**METHODS** Dogs were subjected to shock by repeated bleedings at thirty minute intervals in the following amounts: 1.0% of body weight, 1.0%, 0.5%, 0.5%, and successive bleedings of 0.25% until the blood pressure remained below 60 mm Hg. One kidney was removed one hour after anesthesia with nembutal (32 mg/kg) and prior to commencement of bleeding, and the second kidney was removed after the blood pressure had remained below 60 mm Hg for one hour, or less if the animal did not appear likely to survive the full hour. The oxygen consumption of kidney slices and homogenate was measured in Warburg manometers at 37°C. All determinations were made in duplicate and variations between duplicate experiments were within 10%. The experimental period was one hour.

Experiments with slices were performed in an atmosphere of oxygen and 150 mg of tissue were used in each vessel. The substrate was *l* glutamic acid in a final concentration of M/100. Krebs' Ringer phosphate buffer, pH 7.4, was used.

In experiments with homogenate, 200 mg of tissue in M/10 phosphate buffer, pH 7.8, were used, and the atmosphere was air. The substrate was *dL* alanine in a final concentration of M/20. All vessels contained arsenious oxide in a concentration of M/1000 in order to diminish the residual respiration, since Krebs (3) has shown that arsenious oxide in this concentration eliminates the "sparring action" by depressing cellular respiration and blocking the oxidation of more readily oxidizable substrates including the keto acids.

<sup>1</sup>This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Vanderbilt University.

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formed as a result of the deamination of amino acids. It did not appear to be entirely effective in the experiments in which slices were used. All vessels were made up to a final volume of 2 cc. with buffer.

**RESULTS AND DISCUSSION** Results are presented in table 1. In all animals except one the oxidation of added *d* and *l*-amino acid was suppressed following hemorrhage. With kidney slices the oxygen consumption due to added *l*-glutamic acid was suppressed an average of 28.6% with kidney homogenate the oxygen consumption due to added *dl*-alanine exhibited a similar, but smaller,

TABLE 1

Oxidation of *l*-glutamic acid and *dl*-alanine by slices and homogenate of kidney from dogs before and after shock

NO.	DURATION OF SHOCK	BLEEDING % BODY WEIGHT	mm <sup>3</sup> O <sub>2</sub>					
			Slices + AmO <sub>2</sub>		Homogenate + AmO <sub>2</sub>		% change	
			Before shock	After shock	Before shock	After shock	Slices	Homogenate
3-13-44	min.	3.0	57	48			-19.3	
3-24-44	30	1.5	18	19	205	209	+5.8	+1.9
3-5-44	60	3.8	96	78	270	211	-18.8	-21.8
4-10-44	15	1.2	69	48	453	288	-30.4	-36.8
4-14-44	60	3.9	35	10			-71.4	
4-21-44	30	4.5	38	25	392	361	-31.6	-7.9
Average							-28.6	-16.6

## Control dogs

NO.	TIME BETWEEN SAMPLES	mm <sup>3</sup> O <sub>2</sub>					
		Slices + AmO <sub>2</sub>		Homogenate + AmO <sub>2</sub>		% change	
		First sample	Second sample	First sample	Second sample	Slices	Homogenate
3-20-44	5 hrs. 10 min.			488	421		-8.8
4-11-44	5 hrs.	62	68	326	346	+8.8	+5.8
4-12-44	5 hrs	51	48	261	265	-5.9	-1.5

Value for O<sub>2</sub> consumption of tissue without substrate subtracted.

decrease of 16.6%. In a single instance no impairment of amino acid oxidation was apparent. This animal (No 3-24-44) was bled only 1.5% and showed no evidence of being in shock although the blood pressure remained below 60 mm Hg for the required time. Kidney slices and homogenate from control animals which were maintained under anesthesia for five hours without bleeding exhibited no significant impairment of their ability to oxidize added substrate under similar conditions.

The impaired ability of tissue slices to oxidize amino acids may result from a number of causes. We have shown that the coenzymes, alloxazine adenine

dinucleotide and diphosphopyridine nucleotide may be destroyed in dogs subjected to shock by hemorrhage (4), and that the protein portion of the enzyme may also be destroyed *in vitro* under anoxic conditions (5).

The inhibition of amino acid oxidation by kidney slices is probably not due to the accumulation of metabolites. The oxidation of keto acids is inhibited by arsenious oxide. Lactic acid is freely diffusible so that the dilution while in the

TABLE 2  
*Inhibition of d-amino acid oxidase by serum from shocked dogs*

NO	BLEEDING % BODY WEIGHT	ENZYME WITHOUT SERUM, MM <sup>2</sup> O <sub>2</sub>	ENZYME WITH SERUM, MM <sup>2</sup> O <sub>2</sub>			% INHIBITION*	
			Initial	30 min shock	60 min shock	After 30 min. shock	After 60 min. shock
2-29-44	4.5	192.0	210.7	181.1	172.9	14.0	17.9
3-1-44	2.85	116.5	353.8	332.0	295.9	0	16.25
3-2-44	4.32	183.4	634.0	247.8		61.0	
3-3-44	5.36	131.0	349.7	183.7	167.2	47.6	52.1
3-8-44	1.5	341.9	342.8		209.4†		88.9†
3-10-44	4.5	299.0	365.2	228.3	179.4	37.5	50.8
3-11-44	2.0	299.0	360.2	289.0		19.8	
3-13-44	3.0	298.3	369.2	185.0		49.9	
3-21-44	5.35	400.0	426.0	284.0	242.0	33.3	43.2
3-24-44	1.5	301.5	364.5	343.2	284.3	0	22.6
3-27-44	4.75	380.2	584.0	439.0	310.5	24.85	46.8
4-5-44	3.8	501.0	545.0	482.0	436.0	11.6	20.0
Average						27.2	34.2

Controls

NO	TIME BETWEEN SAMPLES	CONTROL, MM <sup>2</sup> O <sub>2</sub>	INITIAL SERUM MM <sup>2</sup> O <sub>2</sub>	FINAL SERUM, MM <sup>2</sup> O <sub>2</sub>	% INHIBITION*
3-8-44	6 hrs 10 min	384.5	441.0	380.5	13.7
3-14-44	6 hrs 20 min	310.0	366.5	335.0	0
3-28-44	5 hrs 30 min	380.2	499.0	532.0	0
3-29-44	5 hrs 10 min	528.0	637.0	621.0	0
4-11-44	5 hrs	539.0	600.0	577.0	0
4-12-44	5 hrs	539.0	585.0	564.0	0

\* Inhibitions are calculated on the basis of the value obtained with the initial serum. Inhibitions of less than 10% are considered as zero.

† Blood pressure remained below 60 mm Hg for 2 hours and 20 minutes.

Warburg vessels probably results in concentrations too low to account for the degree of inhibition which was encountered.

The inhibition of amino acid oxidation by tissue slices when these have been incubated in nitrogen suggests that tissue anoxia may be a primary causative factor for the reduced ability to oxidize amino acids. Raska (6) has reported a marked decrease in the ability of ischemic kidneys to oxidize amino acids and other substrates. Clark and Rossiter (7) found no change in the ability

of rabbit liver to oxidize alanine after one-third of the body surface had been subjected to burning.

**SUMMARY** Shock induced by hemorrhage markedly decreases the ability of kidney slices and homogenate to oxidize added L-glutamic acid and *d*-alanine.

## 2 INHIBITION OF *d* AMINO ACID OXIDASE BY SERUM FROM ANIMALS SUBJECTED TO SHOCK INDUCED BY HEMORRHAGE

It has been previously reported by various investigators that a decreased ability of tissues to metabolize normally exists in conditions of shock, anoxia, burns, and ischemia. *In vitro* experiments conducted in this laboratory indicate that under conditions of tissue anoxia and cellular damage enzyme inhibitors may be formed. Experiments have been conducted in an effort to determine whether or not such substances are present in the blood of dogs subjected to shock by repeated bleedings.

**Methods** Twelve dogs under nembutal anesthesia were subjected to repeated bleedings by the method described above. Samples of serum were collected at the commencement of each experiment and after the animal's blood pressure had remained below 60 mm Hg for one half hour and for one hour. Six control animals were maintained under anesthesia without bleeding and samples were collected after anesthetization and again after an interval of five to six and one half hours.

Oxygen consumption was measured in Warburg manometers at 37°C in an atmosphere of air. Vessels contained 0.5 cc of enzyme, 0.1 cc of alloxazine adenine dinucleotide, 0.2 cc of *d*-alanine (4.5%), 1.0 cc of serum and M/10 phosphate buffer, pH 7.8, to make the final volume 2.0 cc.

**RESULTS AND DISCUSSION** An inhibitor was found to be present in the serum of shocked dogs which inhibited the oxidation of alanine by the isolated *d*-amino acid oxidase system.

Serum from animals in shock for one-half hour produced an average decrease of 27.2% in amino acid oxidation when compared with the initial sample. Serum collected after one hour of shock produced a 34.2% decrease. In the control series the second sample of serum produced inhibition in only one instance.

The inhibitor is thermostable and the degree of inhibition produced appears to be proportional to its concentration and to the degree of shock which is present. The inhibitor may be lactic acid since lactic acid in concentrations comparable to those found in the serum of shocked animals produced a comparable decrease in the oxidizing ability of the enzyme system. Glucose and pyruvate in concentrations comparable to those found in shocked serum produced no impairment of amino acid oxidation.

It would appear possible that abnormal accumulations of normal intermediary metabolites may constitute a contributing factor to the condition of shock.

**SUMMARY** The presence of a heat stable substance which inhibits the isolated *d*-amino acid oxidase system has been demonstrated in serum from shocked dogs.

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# STUDIES ON SHOCK INDUCED BY HEMORRHAGE

## X HEMOGLOBIN SOLUTIONS AS BLOOD SUBSTITUTES<sup>1</sup>

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In carrying out studies in this laboratory on shock from hemorrhage it was found by Govier and Greer (1) that there is a decrease in liver glycogen, a preliminary rise in blood sugar followed by a fall in some cases almost to zero and an increase in lactic and pyruvic acids in the blood. It was also found by Govier and Greer that in the tissue anoxia known to be present in shock coenzymes are destroyed but that they may be resynthesized by the administration of their respective vitamins (2 3). In spite of such resynthesis of coenzymes the percentage of recoveries of dogs from shock after treatment with vitamins over those of controls was not increased. It was later found that the apoenzyme or protein part of various enzyme systems as well as the coenzyme is inactivated by anoxia (4). Information however is lacking regarding the reconstitution of this part of the enzyme but it was thought that if the tissue anoxia were relieved, aerobic metabolism might be in some measure restored. For this purpose hemoglobin seemed to be an ideal substance.

The old idea that tissue oxidation could not be carried on by hemoglobin outside the red corpuscles (5) was completely disproved by Amberson's classical experiments in which he replaced the blood of animals with oxyhemoglobin salt-sugar solution and his animals lived for many hours in an apparently normal physical and mental state (6) and showed a normal oxygen consumption. More recently Brown and Dale (7) make the following statement regarding hemoglobin solutions. "The blood vessels of a cat's limb can be perfused for hours without losing their characteristic reactions to histamine, acetylcholine and so forth and without an important degree of oedema." There is, therefore, every reason to believe that hemoglobin can carry oxygen to the tissues where normal transfer will take place.

Hemoglobin has certain properties which make it a theoretically ideal substance to use as a blood substitute. In solution it exerts a colloidal osmotic pressure, is an oxygen carrier and can penetrate where red cells cannot. Considering the blood to be made up of 45% red cells and 55% plasma by volume and taking a 7% hemoglobin solution to have an osmotic pressure of 25 mm

<sup>1</sup>This work was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Vanderbilt University.

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Hg (8, 9), which is that of plasma, the hemoglobin contained within the red corpuscles of a given volume of blood made up in a 7% solution with glucose and the proper salts would give a volume approximately four times that of the plasma. That is, for every liter of blood obtained from a donor, approximately 2200 cc of blood substitute could be obtained in addition to 550 cc of plasma, or in the case of man one could add the plasma to great advantage and obtain five times the plasma volume or 2750 cc from 1 liter of blood. If hemoglobin can be shown to be non-toxic the use of such a hemoglobin solution would avoid the waste of discarding four-fifths of the colloidal material of the blood of donors. Hemoglobin solutions will, however, be found to have very different properties from solutions such as gelatin, acacia, or plasma with similar oncotic indices, as will be seen from the following experiments. On this account, hemoglobin solutions can never replace plasma.

Amberson's reviews of the older literature on hemoglobin (10) and the summaries by Amberson as well as Cannon in Mudd's "Blood Substitutes and Blood Transfusion" (11) are so complete that no review of the literature will be given here. A cursory review of the work on hemoglobin will reveal a great difference of opinion regarding methods of preparation, keeping qualities, methemoglobin formation, renal disturbance and general reactions of the patient. From such a review, it is obvious however that as more has been learned about methods of preparation and the handling of hemoglobin the results obtained are very definitely more favorable.

When hemoglobin is made from whole blood without proper care the stroma from broken down red cells may be left behind and cause damage (12). On the other hand, there is certain evidence against this (13). Methemoglobin may also be found in such solutions and kidney damage has been reported from their use, especially in patients with an acid urine (14, 15, 16). On the other hand, stable solutions of hemoglobin can be prepared which do not cause kidney damage (17, 18, 19, 20, 21).

Our experiments were not designed to study hemoglobin as a blood substitute, which is a very elaborate problem, but, as stated above, to see if its use in shocked dogs would have a more beneficial effect than other colloidal solutions or possibly whole blood, as hemoglobin in solution can penetrate where red cells cannot.

We have given hemoglobin solutions to dogs shocked from hemorrhage and have obtained an immediate increase in oxygen consumption of one hundred per cent, and even more.

**METHODS** Dogs were used in these experiments. They were anesthetized by the intravenous injection of 0.032 gm./kg. of sodium pentobarbital which was dissolved in 10% alcohol in 6.4% concentration. This was followed by an injection of 25 cc./kg. of Abbott's heparin solution. They were usually shocked by bleeding at 50 mm. Hg for one hour and then at 30 mm. Hg for one half hour by a method devised by one of us (22). This consists of cannulating a femoral artery and connecting the cannula by means of a rubber tube to a collecting bottle in which 3 cc. of Heparin solution is placed. The bottle is then set at a

level above the dog's heart, such that the height of the column of blood in the connecting tube will maintain the desired pressure. When the artery is opened blood will run up into the bottle and the animal's blood pressure will fall after a few seconds to the desired level. The animal will continue to put out blood for some time however, and then an equilibrium will be reached. After this blood may flow in or out of the animal in small amounts but the pressure will remain very constant. All animals will not stand an hour's exposure to 50 mm Hg as well as a 30 minutes' exposure to 30 mm Hg which is evidenced by a progressive fall in blood pressure toward the end of the period of bleeding. In comparative work such animals must be discarded but as qualitative studies only are considered here some of these animals were included.

Before bleeding, the animal was attached by means of a tracheal catheter with a Waters-Guedel cuff to a Sanborn metabolismeter and the rate of oxygen consumption recorded continuously throughout the experiment.

We have used ox dog and human hemoglobin. Ox hemoglobin was prepared by the method of Amberson et al (6) crystalline dog hemoglobin by the methods of Heidelberger (23) and of Bing (16), both of whom used toluene to produce hemolysis of concentrated suspensions of red cells. Crystalline human hemoglobin was prepared by the method of Cannon and Redish (24). In cases where hemoglobin solution from human and ox blood was prepared by laking the cells in distilled water, the solution was dialyzed against distilled water to remove potassium without further purification. In the case of dog blood the potassium is so low that it can be disregarded. All of these preparations give qualitatively the same results.

We have also used laked whole blood and we have caused *in vivo* laking by injecting distilled water intravenously as described by us elsewhere (25). Finally we have used hemoglobin solutions made by defibrinating dog's blood freezing in dry ice thawing adding 0.9 gm of NaCl per 100 cc. of the water to be added, centrifuging and then adding distilled water in an amount equal to 1.75 times the volume of this hemolyzed stroma free blood. This gives an approximately isotonic solution containing about 6 grams of hemoglobin in each 100 cc. of plasma and water. This procedure has very little effect on the oxygen carrying capacity of the blood.

**OXYGEN CONSUMPTION.** Two illustrations of this are given in the following protocols. In the first hemoglobin Ringer Locke solution was used in the second, hemoglobin in plasma. We have studied the oxygen consumption in shock in 18 dogs using hemoglobin prepared by different methods and obtained from different species, and in every case there was an immediate increase in oxygen consumption after the injection of hemoglobin.

**BLOOD AND PULSATILE PRESSURE.** Amberson (6) reported an increase in blood pressure after the injection of hemoglobin-Ringer Locke solution in animals but this was not very great. Bayless (13) removed about one-third of the calculated blood volume of a cat and replaced this with an equal volume of gum-saline. The blood was then hemolyzed by freezing strained and re-injected. The intravenous injection of 18 cc of blood so prepared into a cat of 1.9 kilograms

weight had no other result than a slight permanent rise in the blood pressure." Buttle, Kekwick and Schweitzer (26) in a comparison of blood substitutes in acute hemorrhage used "a solution containing 3.7 gm of ox hemoglobin per 100 ccm (known as 25% haemoglobin-Ringer)." They make the statement, "The immediate recovery of the blood pressure was good, but there was a tendency for it to fall during the period of observation and to be highly unstable." They conclude in their comparison of different blood substitutes "that plasma is the only one which, in the cat, consistently gives results approximate to those obtained with whole blood. The other substitute solutions we place in the following descending order of value serum, haemoglobin-Ringer, gum saline, red cells in crystalloid solution, isotonic saline, isotonic glucose."

TABLE 1

HUMAN HEMOGLOBIN IN RINGER, DOG 14 KG		CRYSTALLINE DOG HEMOGLOBIN IN PLASMA, DOG 18.2	
Time	Metabolic rate cc/kg/min	Time	Metabolic rate cc/kg/min
2 55	6.4	10 10	5.9
3 10 Bleed at 50 mm Hg		10 25 Bleed at 50 mm Hg	
3 20	3.8	11 25 Bleed at 30 mm Hg	
4 10 Bleed at 30 mm Hg		11 25	4.3
4 40 Stopped bleeding		11 33	3.1
Total blood loss 5% of body weight		11 45	3.1
		11 53	3.1
4 45	2.4	11 55 Stopped bleeding	
4 47 140 cc hemoglobin solution		Total blood loss 4.1% of body weight	
5 04	5.9	12 05 Hemoglobin solution 170 cc	
5 04 125 cc hemoglobin solution		12 10	5.0
5 09	5.9	12 18	6.5
5 09 225 cc hemoglobin solution		12 26	6.2
5 15	5.9	12 45	6.2
Increase in metabolic rate = 140%		Increase in metabolic rate = 109%	

Our experiments with hemoglobin in dogs shocked by hemorrhage gave much more striking changes in the blood pressure and pulse pressure. Hemoglobin solutions not only raised the blood pressure more than gelatin solutions of approximately the same oncotic index, but maintained this pressure for longer periods. In many instances, a moderate injection of hemoglobin (50 cc of 7%) caused a gradual increase of pressure during a period of an hour or so and a single injection might keep the pressure up for several hours. More frequently, however, after a prolonged rise the pressure would gradually fall, but it could be always raised by another injection of hemoglobin solution. The effect on the pulse pressure was very striking, an enormous increase often occurring. Although a mercury manometer was customarily used this same increase was

seen when a glass capsule manometer was substituted. Illustrative curves are given. Although the rise of blood pressure with hemoglobin is greater than that seen with gelatin the striking thing about hemoglobin is the gradual increase in blood pressure over a long time and its prolonged effect. We have as yet made no attempt to analyze these blood pressure and pulse pressure effects but the fact that they are very much less when carbon monoxide hemoglobin is used and still present with recrystallized hemoglobin would indicate that they

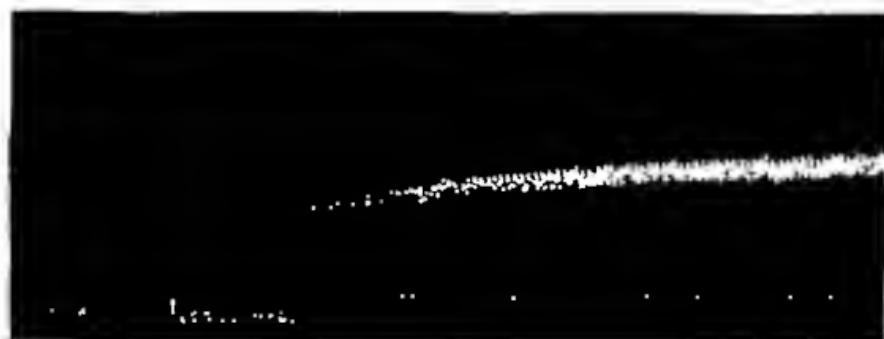


FIG. 1. DOG SHOCKED BY BLEEDING FOR 1 HOUR AT 50 MM. Hg THEN FOR 1/2 HOUR AT 30 MM. Hg.

The pulse became barely perceptible. The characteristic effect of hemoglobin is shown by the gradual and then sustained increase in blood pressure with an increase of pulse pressure.



FIG. 2. A dog was bled rapidly from an artery until heart beat could not be detected (4.45% of body weight). One hundred cc. of this blood was taken in 300 cc. of distilled water 2 gm. of sodium chloride was then added and the mixture injected.

are due in part to better oxidation rather than to a blood pressure raising factor other than hemoglobin or to the oncotic effect only of the solution.

When large amounts of thiamin alone or together with nicotinic acid, riboflavin and ascorbic acid are injected intravenously after a moderate dose of hemoglobin there is an initial fall of blood pressure and then the hemoglobin rise continues. At times this appears to be more rapid and greater than with hemoglobin alone but the end result seems to be the same.

EFFECT OF HEMOGLOBIN IN HEMORRHAGE AND IN SHOCK FROM HEMORRHAGE. After treatment with hemoglobin many of our dogs came out of anesthesia after about 8-10 hours and made very rapid recoveries. They were up and around,

behaving in a normal manner, but almost all died during the night. We have made no attempt to compare statistically the percentage of recoveries of dogs shocked by hemorrhage and treated with hemoglobin, with controls treated with whole blood, salt solution or gelatin. Such studies may give very misleading results unless large numbers of animals are used, and shock of different degrees of duration and intensity studied as well as series of animals treated with different amounts of hemoglobin at various intervals.

At present hemoglobin from the ox, dog or man is used indiscriminately. Hemoglobin solutions of very heterogeneous composition are also used. All of these experiments add to our knowledge, but it is obvious that work must be done on a quantitative basis with substances of known composition.

There are all degrees of hemorrhage and of shock, and blood substitutes of almost any sort will save a certain percentage of animals in the early stages which would have died without such treatment. On the other hand, in more severe cases the purely physical properties of a blood substitute will not be sufficient to save an animal. We have used a large number of dogs in these hemoglobin experiments and can raise the blood pressure and pulse pressure to any desired level as rapidly as desired with hemoglobin solution and maintain this blood pressure level indefinitely. By using the simple method of connecting the femoral artery by means of a cannula and rubber tube to a pressure bottle containing the hemoglobin solution it is possible to obtain any desired blood pressure by raising the bottle to the necessary height. The solution will flow into the animal until the blood pressure equals that produced by the column of blood to the bottle. This pressure will be maintained very exactly as the solution will flow in or out of the animal, depending on the animal's condition. In such a way, the value of various blood substitutes in maintaining blood pressure can be compared by the rate at which fluid is taken up. After five to eight hours of such treatment with hemoglobin at a normal blood pressure of 120 mm., dogs will come out of anesthesia, be up and around, drinking water and behaving in a normal manner. Then within an hour they may die. We have also given hemoglobin by intravenous injection in various concentration, various amounts as well as in large single doses and repeated small doses. We have given sugar, vitamins, certain amino acids, and many other substances with hemoglobin and yet were unable to consistently save shocked animals. On examination, the blood sugar will be found to have fallen to zero in some of these apparently well animals. In others, however, it will be found to be at the normal level, or above this. Thus, there are metabolic disturbances taking place which prevent the animal's complete recovery which blood substitutes alone will not remedy. This in no way detracts from the necessity of finding the best possible blood substitute.

On the other hand, hemoglobin is not toxic. It will save dogs which have been bled until the heart has almost stopped if it is given at once. This we have done many times. Dogs may even be revived with hemoglobin after the heart has stopped, but in the few in which this was tried all died during the night, which might be expected without later treatment with whole blood. However,

if shock is produced in dogs by the method described above, even though less blood is shed than in these cases of *acute* hemorrhage they can not be saved with hemoglobin

It is possible to use hemoglobin in salt-sugar solutions or in plasma, or to make a mixture of hemoglobin red cells and plasma which has an oxygen capacity far above normal. We have carried out a few experiments with these

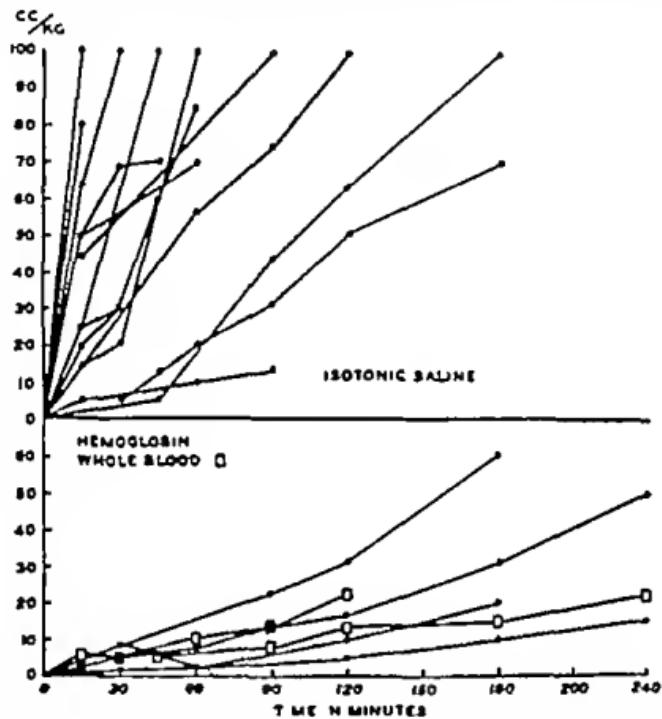


FIG. 3. The figures show the rates of intake by dogs of saline or hemoglobin solutions following a 60 period of bleeding at 50 mm. Hg.

The dogs which received an intra arterial infusion of saline had an average blood loss of 4.6% of their body weight. The infusion bottles were set at 80 mm. Hg pressure for the first 30', then at 100 mm. Hg for the next 30-60', after which they were raised to 120 mm. Hg.

The dogs which received an intra arterial infusion of hemoglobin solutions had an average blood loss of 4.9% of their body weight. The infusion bottles were set at 90-100 mm. Hg for the first 30' after which they were raised to 120-130 mm. Hg.

last two solutions and are of the opinion that they have an even greater effect on oxygen consumption than simple hemoglobin salt-sugar solution

It is with regret that we are unable to give a definite answer based on statistical data as to whether hemoglobin solutions will be found to be more efficacious in the treatment of shock than other blood substitutes. In order to answer this question pure hemoglobin made from the blood of the species to be studied should be prepared. It should be made up in solutions containing various salts and dextrose in various concentrations buffered at the optimum hydrogen ion concentration and given in different concentrations and amounts at various

intervals. If given with plasma, the plasma of homologous animals should be used. Such experiments involve a greater expenditure of funds than are at present available. It would, however, seem reasonable to believe that it would

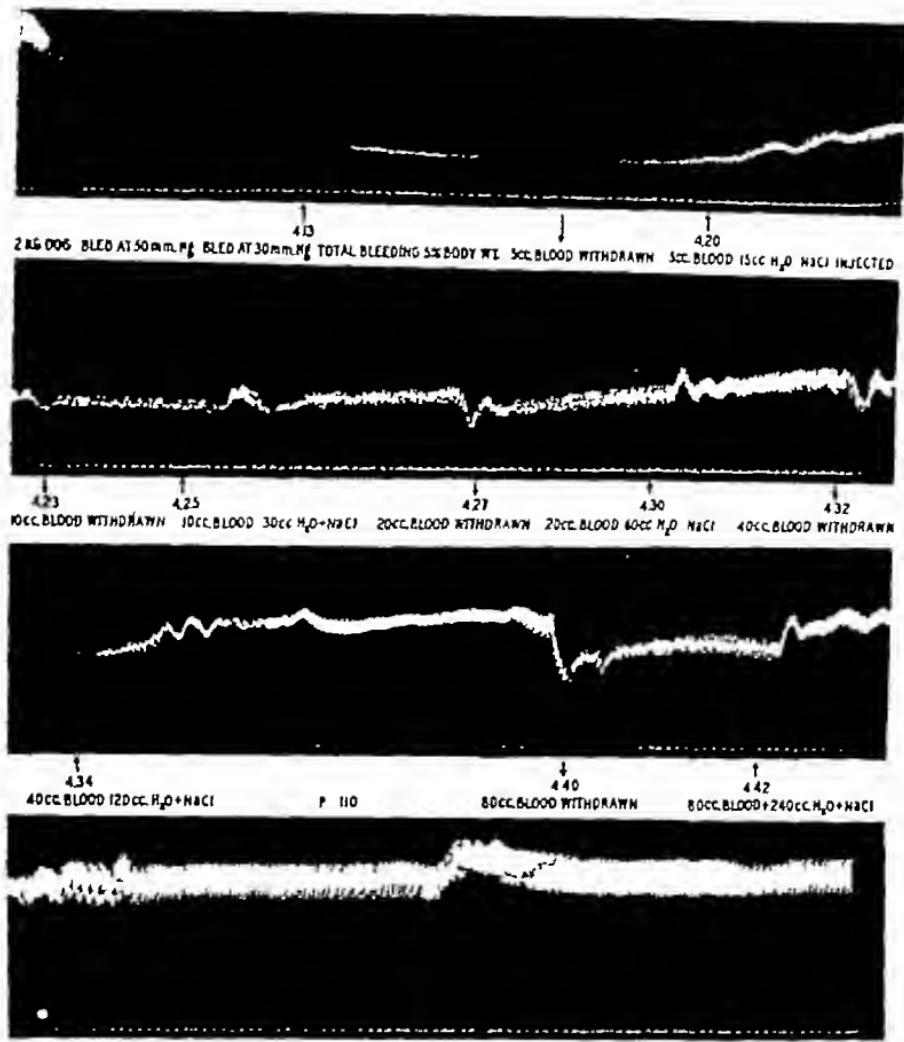


FIG. 4. These curves illustrate how the blood pressure after hemorrhage can be raised by hemolyzing the dog's own blood. This extravascular method allows exact control of the degree of hemolysis which is impossible by intravenous injection of distilled water. An anticoagulant must be used. At 4.47 the effect of vitamins is shown as described on page 6. The weight of the dog was 12 kg and not 2 kg as shown in Fig. 4.

be safe to give hemoglobin plasma solutions experimentally in man. Such solutions can be much more readily made as described above than hemoglobin Ringer solutions, and should be as, or more, efficacious as a blood substitute. In figure 4 the effect after hemorrhage of increasing doses of hemoglobin is

shown. A heparinized dog was used and after the blood pressure had been lowered by bleeding a small amount of blood was drawn into a syringe containing three times this volume of distilled water. Leaking takes place rapidly. An amount of concentrated salt solution sufficient to make the solution isotonic as far as electrolytes were concerned was then drawn into the syringe and mixed with the hemolyzed blood. This mixture was then reinjected. In this manner, the blood pressure can be built up with the animal's own blood to any desired level. Assuming that those are right who maintain that stroma cause no damage such a procedure might be used where blood substitutes are unavailable if it were not for the possibility of intravascular clotting if an anticoagulant is not used and it is unlikely that in an emergency one would be available.

#### CONCLUSIONS

Hemoglobin in Ringer Locke solution, in plasma or in whole blood diluted with salt solution can be made to give solutions with the same oncotic index as blood.

A hemoglobin blood substitute solution differs from gum saline or gelatin solutions as well as from plasma in being an oxygen carrier, and in having a blood pressure raising power which cannot be accounted for by its oncotic properties.

Hemoglobin solutions cause a very marked increase of pulse pressure in dogs shocked by hemorrhage or in dogs after a single large hemorrhage before the onset of shock.

Hemoglobin solutions cause an immediate increase in oxygen consumption of 100% or more in dogs shocked from hemorrhage.

Hemoglobin solutions properly prepared do not seem to be injurious to dogs.

Dogs may be saved from very severe hemorrhage by the injection of hemoglobin solutions.

Dogs cannot be saved by the injection of hemoglobin solutions after severe shock has developed. Neither can they be saved when both hemoglobin and vitamins are given. It is felt that in severe shock from hemorrhage such radical metabolic changes have taken place that the administration of fluid alone in the form of an iso-osmotic solution will not save any large percentage of animals.

It is possible that substances, such as hemoglobin which increase the metabolic rate in cases of shock may actually shorten the survival time. We have found this to be the case if dinitrophenol is given.

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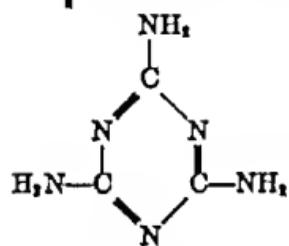
# THE MODE OF ACTION OF THREE NEW DIURETICS MELAMINE, ADENINE AND FORMOGUANAMINE

WERNER L. LIPSCHITZ AND EDGAR STOKEY

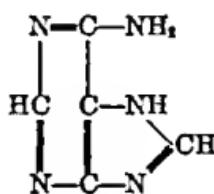
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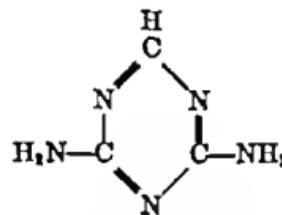
In a previous paper (1) it was shown that among seventy amides, amines and related compounds tested three proved to be potent diuretics. These are melamine adenine and formoguanamine. As shown by the formulas they are related to urea and the xanthines chemically in so far as they contain the group  $\text{—N—C—N—}$  several times in their molecule



Melamine



Adenine



Formoguanamine

Tested with the rat assay method (2) their activities related to urea as the reference standard are 76.5 189 347 respectively. Since these compounds compared favorably with the xanthines diuretics and the organic mercury compounds concerning potency and degree of toxicity they have been further investigated.

1 DIURESIS ON THE DOG Four dogs were used for testing the diuretic activity of these substances and of urea

**Procedure** Several weeks before the beginning of the experiments the external urethral opening in female dogs was exposed through a Falek incision (3). The bladder was drained by catheterization, the urinary excretion was followed before and after administration of the drugs and the volume was calculated for 5-minute periods. In the urine chloride was analyzed with Van Slyke's method (4) or a micromodification (5) of it and total N by Kjeldahlization. In several experiments the hematocrit values were estimated in triplicate in the oxalated blood from the ear and Cl and total N were measured in the plasma obtained from a mixture of 50 mg of powdered sodium oxalate and 2 cc of blood from the saphenous vein under paraffin. The dogs were fasted for 18 hours previous to the experiments but had access to water. In the morning this too was withdrawn, the bladder was emptied and the normal urinary excretion was followed for 2-3 hours. The drugs were then given orally either in 1-3 tablespoonsfuls of minced meat or dissolved or suspended in 50 cc of saline and introduced by stomach tube.

In most instances the peak of diuretic action was reached 1 to 3 hours after administration and the urinary excretion returned toward normal between the 4th and the 6th hour. In other instances, and especially when high doses of

any of these diuretics or of urea were given, the action of the diuretic was still strong after 6 hours. The urinary excretion in control experiments when nothing or two tablespoonsfuls of meat and 50 cc of saline ( $=6$  cc/Kg) were given, is shown in table 1.

All three of the new diuretics, melamine<sup>1</sup>, adenine<sup>2</sup> and formoguanamine, increase the output of water as well as of NaCl in the urine, but not always to the same degree. In some instances the salt concentration in the urine rose considerably, but the concentration in coloring matter and in nitrogen was decreased. On the morning following the administration of the drug the Cl output as well as the Cl concentration in the urine was lower than that before-

TABLE 1

DOG NO & WEIGHT kg	DATE	TIME	URINE EXCRETED cc	URINARY EXCRETION cc./5 min
7 93	11/11/1943	8 45		
		9 45	11 6	0 07
		10 45	10 2	0 85
		11 45	7 2	0 60
		1 50	9 6	0 38
		2 50	6 0	0 50
8 42	4/28/1944	8 45		
		9 45	14 2	1 18
		10 45	13 5	1 13
		10 50	Two tablespoonsfuls of meat + 50-cc saline	
		11 45	13 4	1 12
		1 45	28 4	1 18
		2 45	13 5	1 13
		3 45	13 9	1 16
		4 45	13 9	1 16

the diuretic was given. No significant changes were observed in the composition of the blood or plasma at the height of diuretic action of melamine, the only consistent change being the increase in the amount of red cells per volume of blood the next day, although during the night the dogs were allowed to drink water freely. One representative experiment for each diuretic is shown in figures 1-3.

<sup>1</sup> Melamine was examined in pharmacological experiments by Hesse and Taubmann (6). They found in a dog fed 100 mg./kg. melamine a decrease in blood sugar concentration. But since no other component of the blood was studied it is not clear whether this is a specific effect or an indication of hydremia.

<sup>2</sup> Adenine has been used in therapy of agranulocytosis (7), in doses of 1-2 g. intravenously. Besides this, diuretic experiments with the combination caffeine and adenine were mentioned briefly by Macht and Schroeder (8). The blood pressure of the rabbit is very little affected by adenine (9). Ribosides of adenine and other purines which have some diuretic activity on rats were studied recently by Haas (10).

In the case of melamine the excretion of the drug itself was investigated. The crystalluria occurring after larger doses in rats or dogs was found to be

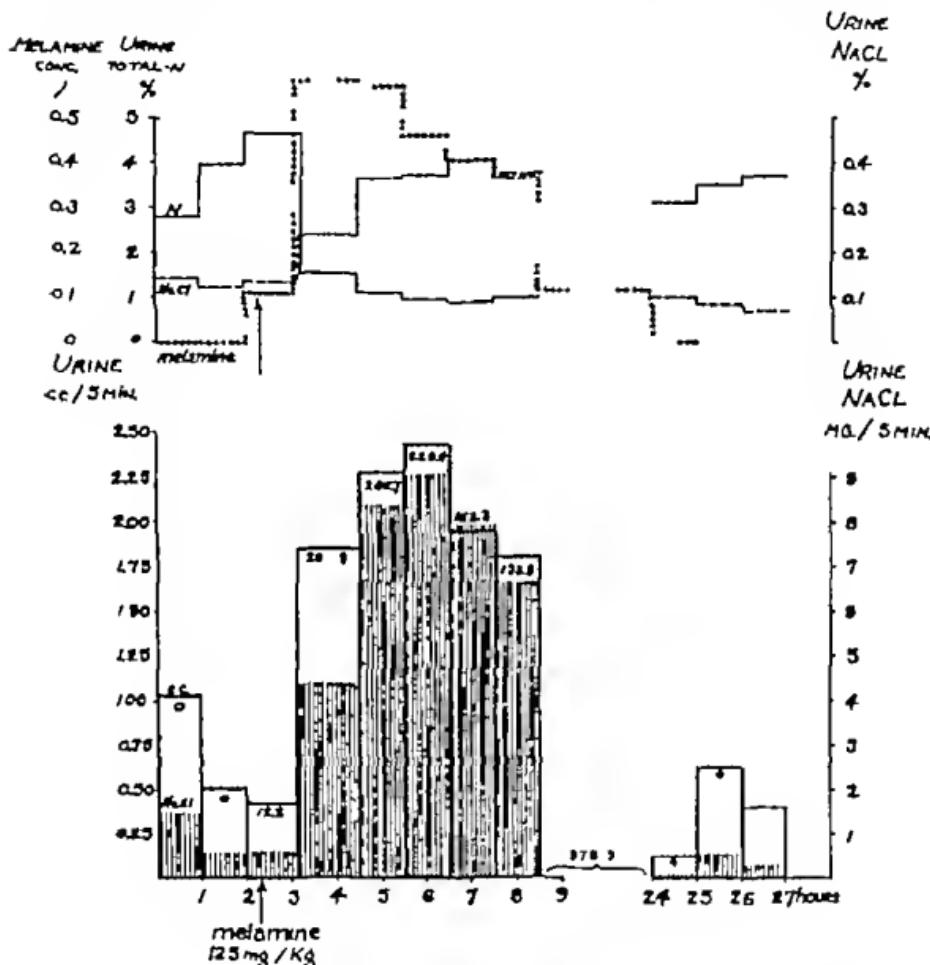


Fig. 1. Dog No 4 7.98 kg 11/15/1943. Fed 1000 mg melamine. The figures in the columns indicate mgs of melamine oxalate found in the urines totaling 1482 mg. These correspond to 865 mg base = 86.5% of the drug recovered. Between 9 and 24 hours 100 cc water were drunk and 194 cc urine excreted. The melamine curve indicates the concentrations in which the drug was excreted in the urines.

	before melamine			during diarrhea			next morning	
Hematocrit figures	38.5	35.5	36.5	35.0	35.0	35.0	43.0	
Plasma total N	0.939			0.902	0.954	0.927	0.967	
Plasma NaCl	0.604			0.630	0.610	0.617	0.619	

due to the excretion of the rather insoluble dimelamine-monophosphate. The total of the excreted melamine can be obtained by adding to the warm urine powdered oxalic acid to a final concentration of 1 per cent. Thus the still

mg/kg melamine, as one per cent solution in saline. They excreted in 6 hours 219.6 cc of slightly acid urine = 131.6% of the fluid (166.9 cc) administered. The urine was boiled with charcoal, filtered and cooled overnight. 391 mg crystals as fine white needles were isolated and twice recrystallized from 200 parts of hot water. Dimelamine monophosphate. Anal. calcd for  $C_6H_{15}O_4N_{12}P$  (350.14) C, 20.56, H, 4.32, P, 8.86. Found C, 20.68, H, 4.49, P, 8.16.

To the filtrates oxalic acid was added up to 1 per cent and the mixture was cooled. The crystals, 130.8 mg, were twice recrystallized from about 200 parts of hot 1% oxalic acid solution, and the silky fine needles were washed with cold water. Monomelamine-monoovalate. Anal. calcd for  $C_6H_8O_4N_6$  (216.01) C, 27.77, H, 3.73, N, 38.88. Found C, 27.23, H, 3.16, N, 38.87. Total melamine recovered in 6 hours 83.4 mg = 50 per cent of the amount fed.

*Excretion of melamine in the urine of dogs.* Dog No 1, 5510 g, fasted overnight, was fed 125 mg/Kg melamine. By treatment of part of the urine collected in 5½ hours with oxalic acid, isolation of the precipitate and recrystallization from hot 1% oxalic acid solution by aid of charcoal 200 mg of long, fine white needles were isolated. Monomelamine-monoovalate. Anal. calcd for  $C_6H_8O_4N_6$  C, 27.77, H, 3.73, N, 38.88. Found C, 27.11, H, 3.39, N, 38.31.

Dog No 3, 4880 g, fasted overnight, was fed 610 mg = 125 mg/kg melamine. The urine excreted in 6 hours was collected by catheterization and yielded after cooling 130 mg crystals which were recrystallized three times from hot water. Dimelaminemonophosphate. Anal. calcd for  $C_6H_{15}O_4N_{12}P$  P, 8.86. Found P, 8.53. From the filtrates 56.1 mg melamine oxalate were isolated by precipitation with oxalic acid. Total yield in melamine base 374.4 mg = 61.3% of the amount fed.

Dog No 4 was used for 17 experiments done in at least one week intervals over a period of 6 months, with various doses of urea, melamine, adenine sulfate and formoguanamine. The substances and doses were not chosen in systematic order. Increased doses of these diuretics produced distinctly increased diuretic effects. So it was possible to get information on the potency of the new diuretics relative to urea. For this purpose primarily those experiments were utilized in which the main diuretic effect was over in 5-6 hours after administration of the drugs. The total excess of the urinary output for 5 hours over the mean output of the preperiod was plotted against the log dose (in mM/kg) of the particular drug, and the antilog of the distance of the dose action curve from that of urea indicates then the relative potency of the drug for the dog (fig. 4).

An experiment done under similar experimental conditions on the same dog which was fed a large dose of theobromine, 0.72 g/kg = 4 mM/kg was disappointing as were the results on rats with the xanthine diuretics. The excess of urinary excretion over that of the preperiod in 5 hours was only 16.5 cc, and the concentration of coloring matter in the urine at the height of diuretic action was hardly less than before, crystalluria was observed. Since this dose of theobromine is already in the toxic range it could not be increased further.

The figures for the diuretic effects in dogs are more scattered, although obtained from the same individual, than in different groups of rats, and it appears that the rat assay method for diuretics is the method of choice. Nevertheless

the sequence of the diuretics concerning their potency was found to be the same on the dog as on the rat (table 2). This fact seems important because it allows

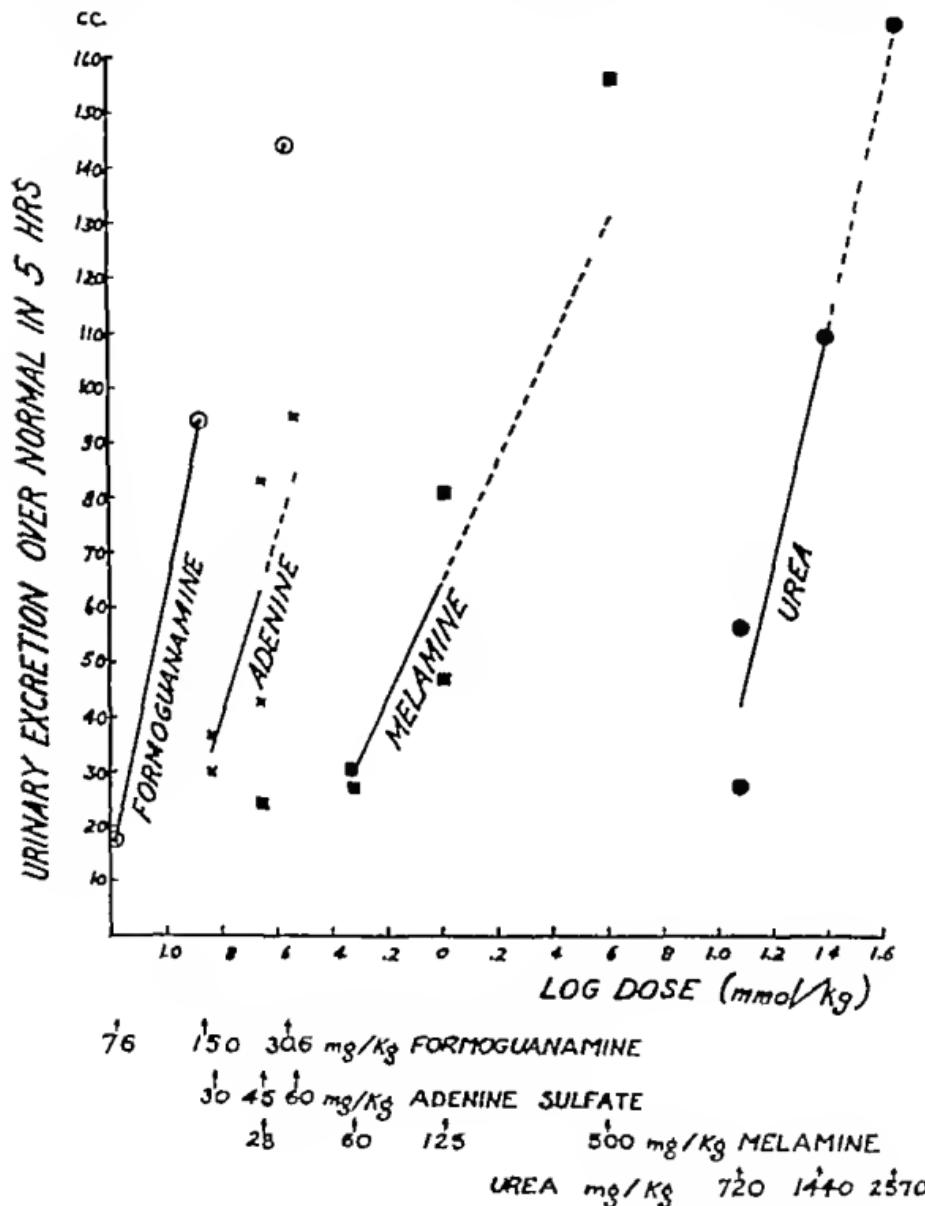


FIG. 4

us to draw conclusions from the results gained with the rat assay method to effects on higher animals and even to therapeutic doses for the patient. The same sequence was seen (2) when the activities of diuretics in common use as

found by this method were compared with the average therapeutic doses employed in patients. This also is shown in Table 2.

**2. MELAMINE ON DIGITALIZED ANIMALS** Preliminary clinical observations with melamine on digitalized patients suggested the possibility that melamine caused a mobilization of digitalis from the depots in the body, and that subsequent doses of digitalis administered were then unexpectedly potent on the circulatory system. Therefore, experiments were performed to throw light on this matter. Experiments on 34 digitalized and 10 normal cats were done. The animals were digitalized by injecting intramuscularly on four successive days the same dose of Digifoline-Ciba. On the fifth day half the number of cats were fed 125 mg./kg. melamine and, 1½ to 4 hours later, following the U.S. Pharmacopoeia technique the lethal dose of standard digitalis powder U.S.P.

TABLE 2

DRUG	DIUREtic ACTIVITY ON		
	Rat	Dog	Patient
Urea	1	1	1
Melamine	76.5	18.6	
Adenine	139	72.5	
Formoguanamine	347	145.0	
Ammonium chloride	2.7		2
Potassium acetate	3.4		2.1
Potassium nitrate	3.9		4.0
Theobromine	7.2		150.0
(Caffeine	32.0		625.0
Theophylline	115.0		480.0
Salyrgan	400.0		1250.0

XII was estimated on these cats as well as on the digitalized control cats. By assaying the powder on normal cats it was ascertained what amount of digitalis was present in the digitalized cats in the Digifoline depot. No significant difference in the fatal dose of digitalis standard powder was found between digitalized cats fed melamine and similarly digitalized control cats.

Since cats in general do not respond readily to diuretics corresponding experiments were done on dogs in which 125 mg./kg. melamine had been proved to be definitely diuretic. The lethal dose of the digitalis standard powder was first determined. In agreement with previous workers (12) it was found that dogs are less sensitive to digitalis than cats, but that preparations of digitalis can be assayed satisfactorily on dogs. The dogs were anaesthetized by subcutaneous injection of 0.01 g./kg. morphine sulfate, and an hour later the digitalis assay was started following the procedure with cats.

The mean lethal dose of the digitalis standard powder for 23 dogs was found to be 1.7 times that for cats. 1 cat unit (C.U.) = 0.56 dog unit (D.U.). This ratio is identical with that of Haskell and coworkers  $\frac{\text{dog dose}}{\text{cat dose}} = \frac{0.56}{1.75} = \frac{4}{5}$ , and that of David and Krishnaswami ratio 1.168 (12). The results on digitalized

dogs with and without melamine are presented in the condensed table 3. Again as in cats no significant difference in the fatal dose of standard digitalis powder on digitalized dogs was found whether or not they were fed diuretic doses of

TABLE 3

EXPERIMENTAL NO.	NUMBER OF DOGS	TREATMENT	DIGITALIS POWDER U.S.P. XII MEAN FATAL DOSE mg./kg.
1	4	None	122.3
	4	Pretreated with total 1 C U = 0.56 D U † Digifoline mean loss in weight 445 g Digitalis depot 0.304 D U = 54.8% of injected	92.1
	4	Pretreated with total 1 C U Digifoline mean loss in weight 420 g Fed 125 mg./kg. melamine 2½ hours before assay	76.5
2	4	None	118.8
	4	Pretreated with total 1 C U = 0.56 D U Digifoline mean loss in weight 195 g Digitalis depot 0.159 D U = 28.4% of injected	99.9
	5	Pretreated with total 1 C U Digifoline mean loss in weight 852 g Fed 125 mg./kg. melamine 2½ hours before the assay	97.5
3	4	None  (Nine dogs in all were injected ½ C U = 0.19 D U Digifoline daily two died on the third day and two more on the fourth day)	118.8
	3	Pretreated with 1½ C U = 0.75 D U Digifoline mean loss in weight 363 g Digitalis depot 0.357 D U = 47.6% of injected	76.4
	2	Pretreated with total 1½ C U Digifoline mean loss in weight 415 g Fed 125 mg./kg. melamine 2½ hours before the assay	60.1
4	8	None	119.3
	4	Pretreated with total 1½ C U = 0.61 D U Digifoline mean loss in weight 388 g Digitalis depot 0.355 D U = 63.1% of injected	73.4
	4	Pretreated with total 1½ C U Digifoline mean loss in weight 420 g Fed 125 mg./kg. melamine 2½ hours before the assay	64.8

C U = cat unit

† D U = dog unit

melamine, and no toxic side-action of the melamine was apparent. Digitalis from a depot in nonedematous animals is not mobilized by melamine.

### 3 THE COMBINATION OF DIURETICS WITH THE ANTIDIURETIC HORMONE.

Divergent results have been reported by Molitor and Pick (13), and Walker, Schmidt, Elsom and Johnston (14) concerning the effectiveness of the anti-diuretic hormone in counteracting xanthine diuresis, and by Fulton, van Auchen, Parsons and Davenport (novasurol) (15), and Walker et al (salyrgan) (14) in counteracting mercury diuresis. Therefore, the question was investigated again on rats, in which diuresis was produced by tap water, large amounts of saline, urea, caffeine, and the new diuretics presented in this paper.

**Procedure** Male rats as used in the diuretic experiments (1, 2) were fasted overnight and, in most instances, also deprived of water. As a rule they were fed 25 cc/kg liquid, and simultaneously 2 cc/kg Pitressin (Parke Davis) diluted with saline was injected so that 10-100 mu/kg were administered. The output of water was determined as prescribed by Burn (16) and also the total urinary excretion in 5 hours measured (2), so that the degree of diuretic response could be estimated. In most instances the rat groups were reused in multiple experiments in order to avoid errors due to a different sensitiveness of the groups.

It was confirmed that water diuresis (tap water, saline) is delayed consistently by doses of pitressin as small as 1 or 2 mu/100 g rat when injected subcutaneously. On the other hand, the diuresis produced by any of the diuretics tested when dissolved in saline, was very little sensitive to the anti-diuretic hormone (table 4).

The fact that Molitor and Pick observed delay of the urinary excretion by the hormone when xanthine diuretics were administered seems to find its explanation in the particular conditions of their experiments. They used dogs which were not only treated with the xanthines but also fed 250 cc (about 30 cc/kg) of tap water. No distinction was made between the diuresis induced by the water and that due to the diuretic. This must be considered in relation to the fact that the dog does not respond well to xanthine diuretics (v. Schroeder [17]). Actually, Wallace and Pellini (18) found a marked antidiuretic effect of xanthines on dogs kept in salt and water equilibrium. The experiments on rats (table 5) demonstrate that the greater the inhibition resulting from the antidiuretic hormone, the greater is the relative effectiveness of water in comparison with the xanthines. The data in Tables 4 and 5 from rats in which pitressin was not used, also show that diuresis produced by urea and the newer diuretics lasts longer than that from water, while caffeine has the shortest action, similarly the figures for the time of the highest rate of excretion are highest with large doses of urea etc., lower for tap water and saline, and again definitely lower for caffeine. Therefore, the possibility that secretion of antidiuretic hormone is the limiting factor for diuresis (19) by xanthines and similar substances (2) is ruled out because, when they were given in saline, the diuresis was not influenced by small amounts of injected antidiuretic hormone. As Wallace and Pellini (18) pointed out long ago, the xanthines seem to be able to produce diuresis only when excessive water is available in the tissues (Lipschitz and Hadidian [1]), and the stimulation of the kidney by xanthine diuretics is small in comparison with other diuretics.

**4. TOXICOLOGY OF THE NEW DIURETICS** For rats the diuretic dose range of the new diuretics has already been reported (1), and much larger doses fed

TABLE 4

Standard procedure: rats fed 25 cc./kg. saline containing the diuretic controls fed 25 cc./kg. tap water. All were given subcutaneously 2 cc./kg. saline with or without Pitressin. Time of highest rate of urinary excretion in minutes.

EXPERIMENTAL NO.	LIQUID FED	SPECIAL EXPERIMENTAL CONDITIONS	MILLIGRAMS/100. PITRESSIN INJECTED						MEAN URINARY EXCRETION IN 5 MIN.	SENSITIVENESS TO PITRESSIN OF THE DIURETIC %	
			0	10	20	40	50	100			
1	Tap water	50 cc./kg. fed	123	+33	+55	+79				+++	
2	Tap water	50 cc./kg. fed	121	+18	+34	+59				+++	
3	Saline	Same rats not deprived of water prevl only	127	+16	+40		+46		58.4	+++	
4	Saline Tap water	50 cc./kg. fed to rats with free access to saline for 20 hrs previous to the exper	133 118	+18	+31		+44		67.9 97.7	+++	
5	Urea Tap water	1.5 g./kg. standard concentrations	97 106	+23	+8		+116		91.6 82.2	0 +++	
6	Urea	1.5 g./kg.	103			+18		-6	79.7	0	
7	Urea	3 g./kg.	143	-7	+3				189.2	0	
8	Urea	3 g./kg.	142			+6	+17		189.2	0	
9	Melamine Tap water	37.5 mg./kg.	96 98	-1	+16		+160		78.7 29.2	(+) +++	
10	Melamine Tap water	37.5 mg./kg.	77 126			+40	+45		79.8 41.0	+	+++
11	Melamine Tap water	200 mg./kg.	158 126	-23	+14		+69		105.4 34.7	0 +++	
12	Melamine Tap water	200 mg./kg.	151 99		+134	+20	-7		108.1 36.9	0 +++	
13	Adenine sulf Tap water	25 mg./kg.	163	+11	-23				54.5 18.1	0	
14	Adenine sulf Tap water	25 mg./kg.	134 83		+91	+47	0		73.5 63.3	(+) +++	

TABLE 4—Continued

EX- PERI- MENT NO	LIQUID FEED	SPECIAL EXPERIMENTAL CONDITIONS	MILLIONITS/KG. PITRESSIN INJECTED						MEAN URINARY EXCRE- TION IN 5 HRS	SENSITIVE- NESS TO PITRESSIN OF THE DIURESIS
			0	10	20	40	50	100		
15	Adenine sulf Tap water	50 mg /kg	181 137	-1 +70	-7				% 89.6 17.8	0 +++
16	Adenine sulf Tap water	50 mg /kg	153 129		+59		+27	+26	80.0 31.2	(+) +++
17	Formoguanamine Tap water	5 mg /kg	136 150	+20 +31	+10				49.5 35.1	0 ++
18	Formoguanamine Tap water	5 mg /kg	146 117		+76		+6	+19	46.2 36.5	0 +++
19	Formoguanamine Tap water	10 mg /kg	171 140	+12 +55	+39				83.8 51.9	+
20	Formoguanamine Tap water	10 mg /kg	214 104		+70	-20	-33		79.0 32.5	0 +++
21	Formoguanamine Tap water	15 mg /kg	172 116	0 +49	-12				95.6 30.9	0 +++

to such animals had no appreciable toxic effects. From this it appears that the therapeutic dose-range of these substances, similar to that of urea, is rather broad (table 6).

Secondly, it appeared from section 1 of this paper that a dog (No. 4), when treated 15 times within 7 months with small, medium or large diuretic doses of all three of the new diuretics successively did not show any untoward effects, except thirst and fatigue at the end of some experiments. There were no significant decrease of weight, or pathological features of the urine or alteration of the general appearance and behavior of the animal. When it was sacrificed and autopsied nothing abnormal was observed in gross appearance or in histological slides from the kidneys, liver, spleen, pancreas and thyroid gland. The bladder showed evidence of chronic cystitis and contained a big stone, but this was attributed to the more than 150 catheterizations.

To enlarge the information on possible toxic effects of these substances they were daily fed to five male rabbits (1 mM/kg. melamine) and three female dogs (1 mM/kg. melamine, 0.173 mM/kg. adenine sulfate, and 0.108 mM/kg. formoguanamine, respectively) for one to four weeks. In several instances the body

temperature of the rabbits was followed for 3½ hours after administration of melamine but only an insignificant rise was noted.

Gross and histological examination of sections taken from heart, lung, liver, spleen, thyroid, pancreas, intestines, kidneys and bladder of the animals did

TABLE 5

EXPER. NO.	LIQUID FED	SPECIAL EXPERIMENTAL CONDITIONS	MILLIGRAMS/200 PITREMIN INJECTED					MEAN URINARY EXCRETION IN 5 HRS	SENSITIVE DOSE TO PITREMIN IN THE DIURETIC
			6	10	20	50	100		
1	Urea Tap water	1.5 g./kg. in tap water	110	+30	+34			72.4	+ (+)
			111		+78			25.0	+++
2	Urea	1.5 g./kg. in tap water	118			+43	+16	89.7	+
3	Urea Tap water	3 g./kg. in tap water	128	+6	+4			154.1	0
			101		+80			61.3	+++
4	Caffeine Tap water Saline	75 mg./kg. in tap water	82	+65	+44			67.3	+++
								84.9	
								20.3	
5	Caffeine Tap water	37.5 mg./kg. in tap water	80	+32	+83			52.2	+++
			116		+61			47.4	+++
6	Caffeine Tap water Caffeine Saline	37.5 mg./kg. in tap water 25 cc./kg. fed 75 mg./kg. in saline 50 cc./kg. fed	90		+61			58.1	+++
			153					57.8	
			147			+9		21.3	0
			153					27.2	
7	Caffeine Saline Tap water	75 mg./kg. in saline 50 cc./kg. fed	137			+22	+2	28	0
								10.6	
								52.3	

TABLE 6

SUBSTANCE	USEFUL DIURETIC DOSE RANGE	MEAN DIURETIC DOSE	LARGEST DOSE FED WITHOUT TOXIC EFFECT	MINIMAL MARGIN OF SAFETY
Melamine	0.1-1.0	0.35	20.0	36
Adenine sulfate	0.062-0.247	0.155	1.55	10
Formoguanamine	0.023-0.09	0.057	2.234	40

not show pathological changes related to the drugs fed (Dr. A. L. Joyner). A zone of fat was found in the inner part of the renal cortex in two dogs but also in the kidneys of three out of four female control dogs of which one was lactating (Dr. F. I. Dessaix).

Several experiments were done with *intravenous* injections of melamine and formoguanamine into cats anaesthetized with ether, blood pressure and respiration were recorded. The effects of epinephrine and histamine were standardized on four cats, following this, they were injected with 0.32 mM/kg melamine, 1 mM/kg melamine acetate in two portions within 6 minutes, 0.33 mM/kg formoguanamine and 2.16 mM/kg formoguanamine dissolved in very dilute HCl, in four portions within 10 minutes, respectively. There occurred neither a drop in blood pressure nor a change in respiration, and the cats afterwards responded normally to epinephrine or histamine.

Since adenine in doses of 1-2 g has been used rather extensively for *intravenous* injections on patients (Reznikoff (20)), and Groppe (9) has shown recently that the blood pressure of the rabbit is very little affected by it, further experiments were not considered important.

#### SUMMARY

1 Melamine, adenine sulfate and formoguanamine, which by the rat assay method were found to be potent diuretics, proved to be active also on the dog. Taking the urea potency as 1 in the dog the activities of the three compounds are in the order of 18.6, 72.5, 145, respectively. These correspond to the rat figures 76.5, 139, 347. The drugs increase the output of NaCl as well as water in proportion to the dose.

2 Melamine is excreted in the dog or rat partly as the crystalline dimelamine-monophosphate. The total can be isolated from the warm urine by precipitation with oxalic acid as the crystalline monomelamine mono-oxalate, and 60-86.5 per cent of the melamine fed to dogs was recovered in the urine in 24 hours.

3 Melamine fed in diuretic doses to digitalized cats and dogs does not change the fatal digitalis dose.

4 Diuresis produced specifically by urea, caffeine, melamine, adenine or formoguanamine fed in saline to rats, is little affected by pitressin—in contrast to water diuresis. The hormone, therefore, cannot be considered as the limiting factor which controls xanthine diuresis.

5 No significant toxic effects of the three new diuretics were found when large doses were administered orally or *intravenously* to rats, rabbits or dogs. This is similar to common experience with urea, and in contrast to the xanthines and the mercury compounds.

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# STUDIES ON SHOCK INDUCED BY HEMORRHAGE

## XI A METHOD FOR THE ACCURATE CONTROL OF BLOOD PRESSURE<sup>1</sup>

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Although "shock" is a symptom complex well recognized by the surgeon, no satisfactory definition has yet been given it, for the very good reason that we do not know what shock is. Not knowing what shock is, one can hardly expect to devise methods for accurately reproducing different degrees of this condition. For the experimental study of shock and its therapy we are offered two methods of approach. In the first, we fix the amount of injury and get varying effects, as individuals vary. In the second, we fix *one effect* of injury and necessarily must vary the amount of injury. In the method here described we fix an effect, namely, a given lowering of blood pressure by hemorrhage.

A cannula is inserted into the femoral artery and connected by means of a rubber tube to a pressure bottle which is set at a height above the dog's heart at which the column of blood to the bottle will support any pressure which one wishes to maintain. When the artery is opened blood will run into the bottle and the blood pressure will fall at once to the desired shock level. Bleeding will continue until an equilibrium is reached and then blood will be found to flow in or out of the animal in small amounts, depending upon the animal's condition, but the pressure will remain constant as long as the animal is able to maintain it. If the femoral cannula is very small, pressures taken from the carotid artery will show slight fluctuations due to the inability of the blood to pass through the cannula rapidly enough to compensate for vascular changes which are constantly taking place, but with a large cannula these variations in pressure are too small to be observed. The animal is heparinized and heparin placed in the pressure bottle.

We have found that time can be saved by using metal cannulae which join by means of a turned tapered joint with a second piece inserted in the end of the rubber tubing.

In our early work, we arranged what we have called a "spill over." That is, the tube from the femoral artery was so arranged that blood would "spill over" at the desired level and none would return to the dog, but this allows considerable variation in pressure and many animals will not last for the desired length of time without a considerable fall of pressure unless blood is allowed to return. We have discontinued the use of this method but have included figures obtained with it for the sake of comparison with our other procedures.

<sup>1</sup> The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Vanderbilt University.

Permission to publish this paper was granted by the Committee on Medical Research on December 26, 1944.

We had hoped that a standard set of figures could be obtained for reference but as our experiments were carried out without due attention to environmental

TABLE 1

*Spill over method—the effect of duration of exposure to a fixed blood pressure*

60 mm. Hg 1 hour	60 mm. Hg 1½ hours	60 mm. Hg 2 hours	60 mm. Hg 2½ hours
<i>Bleeding in % body weight</i>			
4 0 recovered	4 5 died	3 2 recovered	3 7 recovered
3 8 recovered	4 5 died	4 1 died	4 0 recovered
3 5 died	5 4 recovered	4 3 died	5 0 died
3 2 died	2 8 died	4 5 died	3 5 recovered
3 4 recovered	5 25 died	4 5 died	4 0 died
8 7 recovered	3 9 recovered	4 7 died	4 7 recovered
4 1 recovered	4 6 recovered	4 85 died	3 5 recovered
4 8 recovered	2 8 recovered	5 0 died	2 8 recovered
3 5 recovered	3 0 recovered	5 1 recovered	3 85 recovered
3 2 recovered		5 15 recovered	5 1 died
		5 35 died	5 2 died
Average bleeding	3 7%	4 1%	4 3%
Recovery	1/4 = 80%	1/4 = 55.55%	1/4 = 45.45%

TABLE 2

*Bottle method—the effect of various durations of exposure to different blood pressures*

60 mm. Hg 1 hour	60 mm. Hg 1 hour	60 mm. Hg 2 hours	60 mm. Hg 1½ hours 30 mm. Hg 1 hour
<i>Bleeding in % body weight</i>			
2 8 recovered	3 4 recovered	4 15 died	4 55 died
4 8 recovered	4 4 recovered	5 1 died	3 5 died
2 5 recovered	4 5 recovered	4 4 recovered	2 6 died
4 5 died	5 2 died	5 8 recovered	3 5 died
2 5 died	5 4 died	4 0 died	5 0 died
3 3 died	3 5 recovered	4 7 recovered	3 3 recovered
4 8 recovered	4 7 died	4 8 recovered	4 3 died
5 0 died	3 9 recovered	4 4 died	4 4 recovered
4 8 died	4 3 died	4 5 died	5 0 died
5 1 recovered	5 8 recovered	4 6 recovered	4 9 died
			5 3 died
Average bleeding	4 0%	4 5%	4 6%
Recovery	1/4 = 80%	1/4 = 60%	1/4 = 80%
			1/4 = 18.2%

temperatures the results serve only to show the variation in mortality rate with exposures to several pressure levels for various periods of time. Our work was begun in the winter and several hundred dogs shocked by this method during

the spring and summer months. It was found that in July and August, when the temperature of our laboratory reached 105°F almost daily, animals which had withstood an exposure to pressures of 50 mm Hg for one hour and then to 30 mg Hg for half an hour would die before the time was up. Later, many died in less than an hour when exposed to a pressure of 50 mm Hg. These experiments confirm the work of Cleghorn (1) and others who have reported similar effects of temperature on the resistance of animals to shock. It might be well to mention in this connection the work of Govier (2) who found in experiments carried out here that the vitamin content of the diet greatly affected the resistance of dogs to shock from hemorrhage.

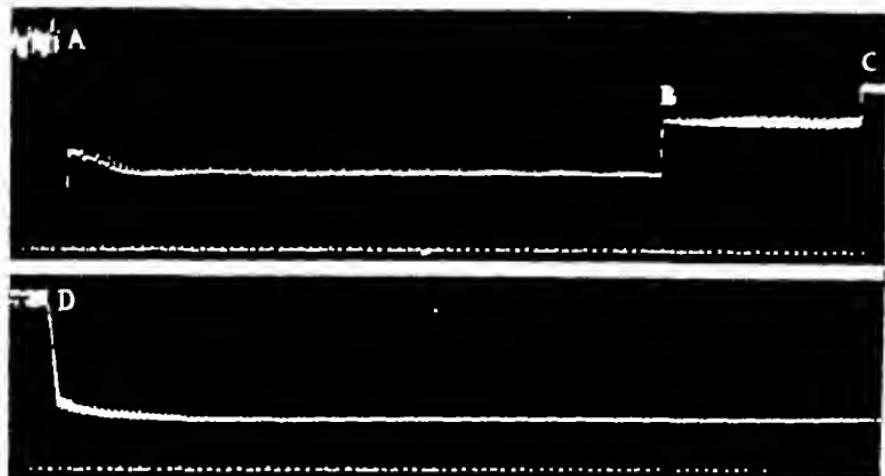


FIG. 1. CURVES FROM TWO DOGS

At A and at D the artery was opened to the pressure bottle. At B and at C the pressure bottle was raised.

It is possible to drop the pressure in stages by setting the pressure bottle at the dog's normal pressure and then lowering it in stages, or one may mechanically lower the bottle at a constant rate so that it will reach the desired level in a fixed time, in which case a smooth curve can be obtained. If one reverses the above procedure and, after the animal has been shocked, places the blood substitute to be studied in the pressure bottle set to give any desired pressure, one may compare the rate at which this solution is taken up by the animal with that of other solutions. The difference in rate of uptake is very striking when different solutions are used, as reported by us elsewhere (3).

#### CONCLUSIONS

A method has been described for the accurate control of blood pressure during the production of shock by hemorrhage.

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# THE COMPARATIVE PHARMACOLOGICAL ACTION OF SOME PHENYL- CYCLOHEXYL- AND CYCLOPENTYLALKYLAMINES

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Since the publication of the results of Barger and Dale's classical investigation of the relationship between chemical structure and sympathomimetic action there have been prepared many new compounds based upon the phenethylamine nucleus and similar in structure to epinephrine but having somewhat different pharmacologic actions. These compounds differ from each other in the number and nature of the groups substituted on the benzene ring and in the number and arrangements of the carbon atoms in the alkylamine side chain.

A few publications have described the pharmacology of compounds in which cyclic arrangements other than the benzene ring have been incorporated into the general sympathomimetic structural pattern. Tainter (1) described the pressor action in cats of  $\beta$ -2-thienylethylamine, and Schulte, Reif, Bacher, Lawrence and Tainter (2) the effect of this substance on the motor activity of rats. Alles and Feigen (3) have described the pressor action of  $\beta$ -2-thienyl and  $\beta$ -2-furylisopropylamine and Gunn and Gurd (4) the pharmacology of cyclohexylmethyl  $\alpha$  and  $\beta$ -cyclohexylethyl  $\beta$ -cyclohexylisopropyl and  $\beta$ -cyclohexenylisopropylamines. The results obtained with these compounds are of considerable theoretical interest and suggest the possibility that organic structures of this type may be of therapeutic value.

A number of cyclohexylalkyl and cyclopentylalkylamines have been synthesized by Blieke and Monroe (5) and Blieke and Zienty (6) and made available to this laboratory for pharmacologic investigation. In the present communication, we have described the actions of ten of these comparing them with the corresponding phenylalkylamines when these were available.

**1. PRESSOR ACTION** Dogs were anesthetized with sodium pentobarbital atropinized and the carotid blood pressure recorded. Injections were made into the exposed femoral vein and the resultant changes in blood pressure compared with those obtained following the injection of epinephrine and Neo-Synephrine. All amines except epinephrine and Neo-Synephrine were dissolved in distilled water to make a 1 per cent solution of the hydrochloride salt. Table 1 summarizes the action on blood pressure. In order to avoid the repetition of chemical names in subsequent discussion the abbreviations indicated in parentheses in this table will be used.

Compound MCH is most active in elevating blood pressure (table 1 and figure 1) being approximately as active as ephedrine about  $\frac{1}{4}$  as active as Neo-Synephrine and  $\frac{1}{8}$  as active as epinephrine. Compounds ECH, MCP

TABLE I  
Amines studied and their action on the heart and blood pressure

COMPOUND	AV DOSE mgm./kgm	AV CHANGE IN BLOOD PRESSURE mm Hg (- +)	AV DOSE mgm./kgm	MAX. CHANGE IN HEART RATE beats/minute (- +)	MAX. CHANGE IN AMPLITUDE % normal (- +)	DURA- TION OF CHANGE minutes
Dimethyl- $\beta$ phenethyl (DMPh)*	0.54 (0.1 0.9)	+14 (-40 +72)	0.31 (0.05 0.50)	+19 (0 +29)	+30 (+26 +33)	4 (1.5)
Ethyl- $\beta$ phenethyl (EPPh)	0.45 (0.1 0.95)	+3 (-30 +33)	0.27 (0.05 0.50)	+36 (+10 +93)	+35 (+14 +54)	7 (3.11)
Diethyl- $\beta$ phenethyl (DEPh)	0.65 (0.5 0.95)	-24 (-14 -44)	0.31 (0.20 0.50)	+14 (0 +21)	+10 (0 +21)	4 (0.10)
Methyl- $\beta$ -cyclohexylethyl (MCHE)	0.48 (0.2 0.8)	+55 (+26 +100)	0.33 (0.10 0.50)	+37 (0 +108)	+43 (+25 +90)	6 (2.14)
Dimethyl- $\beta$ -cyclohexylethyl (DMCH)	0.42 (0.2 0.95)	-7 (0 -30)	0.27 (0.10 0.50)	0	0	
Ethyl- $\beta$ -cyclohexylethyl (ECH)	0.62 (0.2 0.95)	+36 (+18, +50)	0.32 (0.10 0.50)	+34 (+12 +86)	+35 (+21 +50)	8 (3.12)
Diethyl- $\beta$ -cyclohexylethyl (DECH)	0.66 (0.2 1.34)	-19 (-10 -36)	0.29 (0.20, 0.50)	0	0	
Methyl- $\beta$ -cyclopentylethyl (MCP)	0.41 (0.2 0.8)	+34 (+6 +70)	0.20 (0.20 0.40)	+34 (+14 +73)	+14 (0 +25)	3 (3.4)
Dimethyl- $\beta$ -cyclopentylethyl (DMCP)	0.45 (0.2 1.0)	-21 (-10 -38)	0.25 (0.10 0.50)	0	0	
Ethyl- $\beta$ -cyclopentylethyl (ECP)	0.68 (0.43 0.95)	+3 (-8 +20)	0.25 (0.05 0.50)	+15 (0 +27)	+20 (0 +51)	7 (3.9)
Diethyl- $\beta$ -cyclopentylethyl (DECP)	0.78 (0.315 1.43)	-19 (-10 -36)	0.22 (0.20 0.25)	0	0	
Methyl $\gamma$ -cyclohexylpropyl (MCH Propyl)	0.48 (0.2 0.8)	+20 (+10 +32)	0.39 (0.20 0.50)	+2 (0 +9)	+10 (0 +51)	4 (3.6)
Dimethyl $\gamma$ -cyclohexylpropyl (DMCH Propyl)	0.35 (0.2 0.53)	-1 (-14 +10)	0.31 (0.20 0.5)	0	0	
Neo-Synephrine	0.022 (0.008 0.050)	+41 (+12 +92)				
Epinephrine	0.0019 (0.0003 0.0050)	+45 (+30, +80)	0.0025 (0.0005 0.01)	+22 (0 +90)	+60	1 (1.2)

\* All compounds are the amine hydrochlorides

and MCH Propyl are less active and EPh and ECP are least active. Compound EPh is quite unpredictable in action causing rises in about 50 per cent of all experiments, the rise sometimes being preceded by a fall causing only a fall in

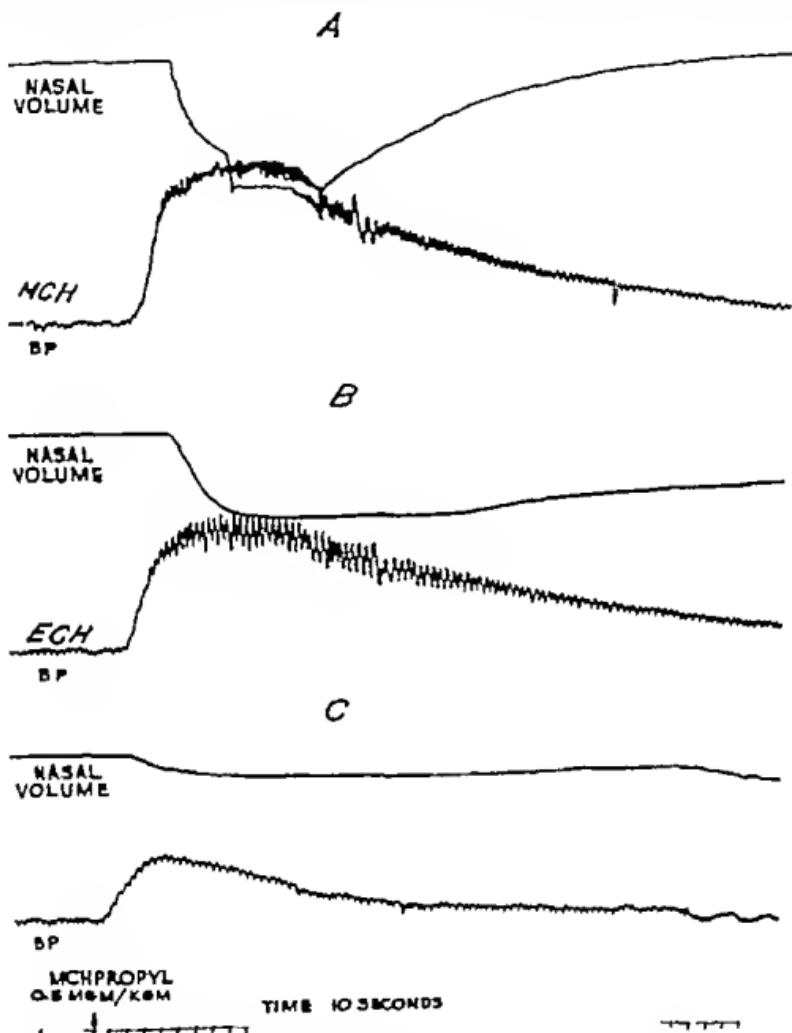


FIG. 1 CHANGES IN CAROTID BLOOD PRESSURE AND NASAL VOLUME IN THE DOG  
The  $\dagger$  indicates the time of injection of the drug. All injections were made into the femoral vein

blood pressure in about 25 per cent of the experiments and is without effect in another 25 per cent. With DMPh there is sometimes a transient rise in blood pressure but as with EPh sometimes only a fall in pressure results. DMCH and DMCP cause transient falls in blood pressure the tertiary diethyl- $\beta$ -cyclohexyl and  $\beta$ -cyclopentylethylamines cause distinct falls in blood pressure

As with ephedrine, cocainization diminished the rise in pressure obtained with the vasoconstrictor compounds. In this respect they resemble the weak vasoconstrictors studied by Tainter (7). Compounds containing the cyclohexyl ring were found to be somewhat more effective than their corresponding cyclopentyl analogs in elevating blood pressure. Those compounds causing rises in blood pressure did not cause any apparent constriction of the conjunctival blood vessels of rabbits. However, marked shrinkage of the nasal mucosa of dogs resulted from their intravenous injection (figure 1).

**2 ACTION ON THE HEART** Recordings of the action of the left ventricle of 6 vagotomized dogs were made by means of a Cushny myocardiograph. Positive artificial respiration was maintained throughout the experiment. In two other experiments the pulse rate and blood pressure were determined, the chest not being opened or artificial respiration required. All of the secondary methyl- and ethylamines increased the rate and amplitude of ventricular contraction (table 1 and figure 2), the secondary methylamines MCH and MCP being somewhat more active than the corresponding ECH and ECP. Compound MCH Propyl was found to be only weakly active.

All compounds containing the phenyl ring (DMPh, EPh and DEPh) had some action on the heart although DEPh was relatively weak and uncertain in action. The tertiary  $\beta$ -cyclohexylethyl- and  $\beta$ -cyclopentylethylamines were without definite cardiac action. Of the above compounds which stimulated the heart, even those that are weakly stimulating caused more prolonged action than did epinephrine when given in an amount which would cause a comparable amount of initial stimulation. In this respect, their action most resembles ephedrine. In a few instances with the most active compounds, there was evidence of stimulation for as long as 10 minutes following injection.

**3 ACTION ON RESPIRATION** Respiration in the anesthetized dog was recorded kymographically, the recording tambour being attached to the tracheal cannula. Observations with some compounds were obtained from as many as 8 dogs. Only the tertiary dimethylamines had any significant effect on respiration. With the exception of DMCH Propyl, intravenous injection of 0.1 to 1.0 mgm./kgm. caused distinct increases in both the depth and rate of respiration which lasted for approximately 1 minute (table 2 and figure 3). Arranged in the order of their effectiveness they are DMPh > DMCP > DMCH > DMCH Propyl. In a few instances the hydrochloride salts of straight chain dimethylamines were tried, as above indicated. Butyldimethylamine was without any apparent effect, amyldimethyl- and heptyldimethylamine caused a transient stimulation, and heptyl- and octyldimethylamine were without effect.

**4 ACTION ON SMOOTH MUSCLE** a) *Small Intestine* Determination of the effects on intestinal motility may be divided into two groups of experiments 1) those on the isolated segment of rabbit jejunum, according to the method of Magnus, and 2) those on the small intestine of anesthetized dogs, motility changes being recorded manometrically from a condom balloon inserted into the lumen of the jejunum. In the first group of experiments the tabulated data was obtained from segments taken from at least 6 rabbits so that for each com-

pound used, data was obtained from a minimum of 6 individuals although several compounds may be tested on each intestinal segment. In the second group 3 dogs were used and observations obtained with those compounds found

A

MCH  
0.25 MG/M KG/M

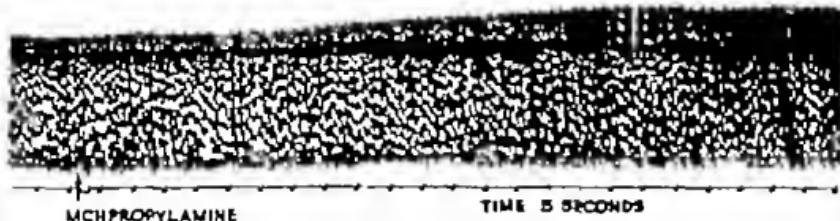
TIME 5 SECONDS

B

ECH  
0.25 MG/M KG/M

TIME 5 SECONDS

C

MCHPROPYLAMINE  
0.25 MG/M KG/M

TIME 5 SECONDS

FIG. 2 EFFECT ON THE HEART

Myocardiograms of the action of the left ventricle of the dog. The  $\uparrow$  indicates the time of injection of the drug. All injections were made into the femoral vein.

to be most effective in raising blood pressure. Table 3 and figure 4 gives the summary of and illustrates the results obtained on the isolated jejunal segment. It should be noted that on the isolated intestinal segment the tertiary dimethyl

within 30-45 minutes and some mydriasis was evident for as long as 2½ hours. Other compounds in this series were without action.

TABLE 3  
*Action on the isolated intestine and uterus*

COMPOUND	ISOLATED RABBIT JEJUNUM		ISOLATED RAT UTERUS	
	Dilution	Response	Dilution	Response
DMPh	1-400,000 1-200,000	C* or C followed by R*	1- 50,000	Decreased tonus and motility in 50% of the rats
EPH	1- 50,000	NA* in $\frac{1}{2}$ —R in $\frac{1}{2}$	1- 50,000	Decreased tonus and motility in 50% of the rats
DEPh	1-200,000 1- 50,000	C and/or R	1- 50,000	Increased tonus and motility in 2 out of 3 experiments
MCH	1-100,000 1- 50,000	R	1- 40,000 1-200,000	Decreased tonus and motility
DMCH	1-200,000 1-100,000	C and/or R	1- 50,000 1-200,000	Marked increase in both tonus and motility
ECH	1-200,000 1-100,000	R	1- 50,000	Decreased motility and tonus
DECH	1-500,000 1-100,000	R	1- 50,000 1-100,000	Marked increase in both tonus and motility
MCP	1-100,000	R		
DMCP	1-400,000 1-100,000	C and/or R	1- 50,000	Increase in both tonus and motility
ECP	1-200,000 1-100,000	R		
DECP	1-200,000 1-100,000	R	1- 50,000	Increase in both tonus and motility
MCH propyl	1-200,000 1-100,000	R	1- 20,000 1-100,000	Moderate decrease in tonus and motility
DMCH propyl	1-200,000 1- 50,000	R	1- 20,000 1- 50,000	Moderate increase in tonus and motility

\* C = increased tonus, R = decreased tonus, NA = no action

5 TOXICITY Acute toxicity was determined by intraperitoneal injection into albino mice. Deaths were recorded for 48 hours following injection. The results obtained are shown in detail in table 4. An examination of this data

suggests that a) saturated ring compounds are more toxic than the corresponding phenyl analogs b) the tertiary dimethyl and diethyl- $\beta$ -phenethyl- $\beta$ -

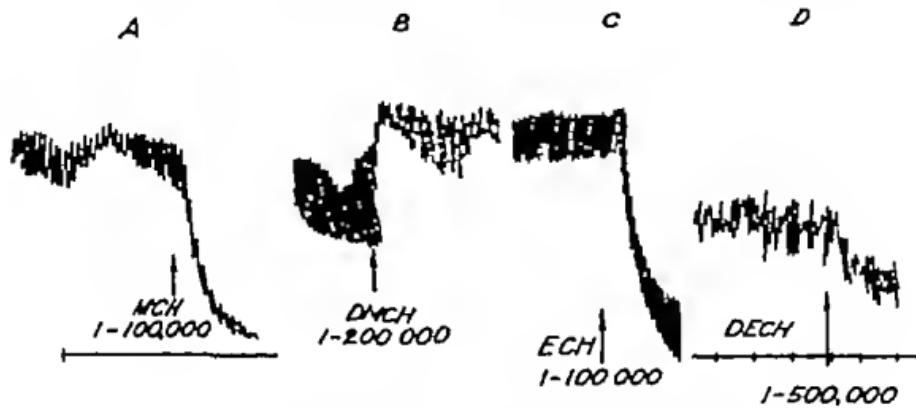


FIG. 4. EFFECT ON THE INTESTINE

Record of the tonus and motility of the isolated jejunal segments of rabbits according to the method of Magnus. The numbers represent the final dilution of the drug in the muscle bath. The  $\dagger$  indicates the addition of the drug to the bath.

TABLE 4  
Acute toxicity in albino mice

COMPOUND	APPROX. DOSAGE LD 50 <sup>a</sup>	DOSE (mgm./kgm.)											
		25	50	65	75	80	85	90	100	110	125	135	150
DMPh	135								0/6 <sup>b</sup>		2/6	3/6	6/6
EPH	125								0/3	0/10	16/40	6/10	13/13
DEPh <sup>c</sup>													
MCH	125												
DMCH	85				0/13			14/40	10/10	3/8			
ECH	100			0/6	1/3				6/30	5/18	4/10	10/10	
DECH	80		0/6		0/6	5/6	6/6						3/3
MCP <sup>c</sup>													
DMCP	85				0/10	3/13		5/10		12/13			3/3
ECP	115					0/3				1/13	4/10	10/10	3/3
DECP	50	0/10	4/10			10/10				10/10			
MCH propyl	125					0/3				2/18		5/10	7/10
DMCH propyl	135								0/3	2/13		0/10	3/10

Intraperitoneally into albino mice

<sup>a</sup> Number dead/number injected

<sup>c</sup> Not determined—insufficient amount of drug

<sup>b</sup> Dose = 115 mgm./kgm.

cyclohexylethyl and  $\beta$ -cyclopentylethylamines are definitely more toxic than the corresponding secondary amines, c) although the data is incomplete, the secondary ethylamines appear to be somewhat more toxic than the secondary

methylamines. There was no significant difference in the toxicity values obtained with methyl- and dimethyl- $\gamma$ -cyclohexylpropylamine.

**DISCUSSION** An examination of the available pharmacologic literature indicates that cycloalkylamines have received little attention as sympathomimetic agents. Waser (8) found an elevation of blood pressure in dogs following the injection of  $\beta$ -cyclohexylethylamine. Gunn and Gurd (4) in a much more extensive study of compounds of this type found that the reduction of the phenyl ring of several sympathomimetic amines did not change the qualitative character of these substances. Their series included only  $\alpha$ -phenethylamine,  $\beta$ -phenethylamine, with their reduced analogs and the following reduced amines, cyclohexylmethylamine,  $\beta$ -cyclohexyl isopropylamine and  $\beta$ -(1-cyclohexenyl)-isopropylamine. The availability of compounds synthesized by Blicke and Monroe (5), and Blicke and Zienty (6) has permitted us to extend the investigation of compounds containing a reduced ring to include secondary and tertiary methyl- and ethylcycloalkylamines not previously described in the pharmacologic literature.

It is difficult to compare pharmacologic results obtained in different laboratories where methods of investigation differ and species of animals used vary somewhat. However, one important difference noted in our blood pressure results with dogs, as compared with those obtained by Gunn and Gurd with cats, is the appearance in our experiments of an important depressor component in response to the intravenous injection of those compounds containing the phenyl ring. This was notably absent from the kymograms obtained with the secondary methyl- and ethyl  $\beta$ -cyclohexylethylamines. Thus, the "average" effect of compound EPh on blood pressure is only slightly pressor due to the inclusion of experiments where this substance caused only a fall in blood pressure. On the other hand, ECH appears to be considerably more active in elevating blood pressure, inasmuch as it invariably caused a rise. We did not have methyl- $\beta$ -phenethylamine available for investigation. However, the action of this substance on blood pressure was described by Tainter (7) who reported essentially the same variable action noted by us with EPh. Methyl- $\beta$ -cyclohexylethylamine was found to be somewhat more pressor than ethyl- $\beta$ -cyclohexylethylamine. This is in general agreement with comparable data on the phenethylamines as reported by Chen, Wu and Henriksen (9). It seems not improbable that MCH is less pressor than  $\beta$ -phenethylamine inasmuch as Gunn and Gurd report this substance to have an epinephrine ratio of 50-100 whereas we found an average epinephrine ratio of approximately 250 for MCH. The substitution of a cyclopentyl for a cyclohexyl group reduces pressor action. Thus MCH is more pressor than MCP and ECH more pressor than ECP. Again, the appearance of a depressor component in ECP considerably reduces the average pressor action. The lengthening of the alkamine side chain to propyl as with MCH Propyl distinctly reduces pressor action. This substance was found to be weaker in action than either MCH or MCP. The vasopressor compounds described here exerted actions on other sympathetically innervated structures qualitatively similar to those noted for sympathomimetic amines.

They cause cardiac acceleration mydriasis relaxation of the small intestine and uterus (rat)

The tertiary dimethylamines appear to have a nicotine-like action. Thus we find that they stimulate respiration and may cause contraction of the smooth muscle of the intestine and uterus. The actions on circulation are more variable. Compound DMPh sometimes increased blood pressure and heart rate whereas DMCH and DMCP lowered blood pressure and were without important effects on the heart rate. The further weighting of the amino nitrogen as in DEPh, DECH and DECP produced compounds that were depressor and with the exception of DEPh were without action on heart rate. They did not alter the respiratory rate. It is interesting to note that although the tertiary diethylamines usually relax the small intestine they stimulate the uterus. These tertiary amines appear not to be sympathomimetic in action.

Direct comparison of the effect of reduction of the phenyl ring on the toxicity of the resultant compound was possible in only one instance (EPh, ECH). The results obtained are in essential agreement with the findings of Gunn and Gurd who reported cycloalkylamines to be somewhat more toxic than their corresponding phenyl analogs. Further examination of the data reveals that secondary ethylamines are more toxic than the secondary methylamines and that the tertiary amines are more toxic than the corresponding secondary amines except in the case of the cyclohexyl  $\gamma$  propylamines. Dimethyl  $\gamma$ -cyclohexyl propylamine appears to be slightly less toxic than methyl  $\gamma$ -cyclohexylpropylamine.

#### SUMMARY

1 The secondary methyl and ethyl- $\beta$ -cyclohexylethylamines and - $\beta$ -cyclopentylethylamines have distinct sympathomimetic activity. They elevate blood pressure, increase heart rate, relax the intestinal musculature and relax the uterus (rat).

2 The secondary methylamines are more effective sympathomimetic agents than the corresponding secondary ethylamines. Methyl  $\gamma$ -cyclohexylpropylamine is less active than either methyl- $\beta$ -cyclohexylethylamine or methyl- $\beta$ -cyclopentylethylamine.

3 The tertiary dimethyl- $\beta$ -phenethyl- $\beta$ -cyclohexylethyl- $\beta$ -cyclopentyl ethyl and  $\gamma$ -cyclohexylpropylamines exert a nicotine like action in that they increase the respiratory rate and stimulate the smooth muscle of the intestine and uterus. Effects on blood pressure are variable.

4 The tertiary diethylamines in this series lower blood pressure and depress the activity of the intestinal musculature. They stimulate the uterus (rat).

5 Where direct comparison was possible ethyl- $\beta$ -cycloalkylethylamines were found to be somewhat more toxic than the corresponding phenethylamine. The tertiary dimethyl and diethyl- $\beta$ -cycloalkylethylamines were found to be more toxic than the corresponding secondary methyl and ethylamine.

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# THE EFFECT OF HEPATOTOXIC ALKALOIDS ON THE PROTHROMBIN TIME OF RATS

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Liver injury due to pathologic processes or toxic agents is frequently associated with hypoprothrombinemia. Of the latter chloroform phosphorus and tannic acid are examples as demonstrated by Smith and his co-workers (1, 2) and Wells, Humphrey, and Coll (3). Previous reports (4, 5, 6) from this laboratory showed that many alkaloids of the *Senecio* species and monocrotaline of *Crotalaria spectabilis* produced necrosis of the mouse's or the rat's liver. It was repeatedly pointed out that sinusoidal congestion and hemorrhage into the cords of cells occurred with the liver necrosis. The present series of experiments was carried out to determine (a) any changes in the prothrombin time of rats following a single intravenous injection of senecionine, retrorsine, pterophine, spartiodine, monocrotaline or scleratine, (b) any similar action possessed by the degradation products of retrorsine and monocrotaline and (c), any antagonizing effect by menadione (2-methylnaphthoquinone), or other dietary factors if hypoprothrombinemia could be demonstrated with the above alkaloids.

The method for prothrombin determination was essentially that of Quick (7). In rats a plasma dilution of 12.5% as advocated by Link (8) was found most satisfactory. Hemagelen (Brain Thromboplastic Suspension, Lilly) was employed as the thromboplastic agent. This product would not yield absolute prothrombin time but it gave relative values suitable for comparative purposes, the chief advantage of which was its stability. Fresh solutions were made by diluting a volume of 1 cc. with 49.5 cc. of 0.9% solution of sodium chloride and 49.5 cc. of 0.023 M solution of anhydrous calcium chloride. Readings of results were made in front of a beam of strong light. The mean (geometric) prothrombin time observed on 26 normal rats was  $39.8 \pm 0.7$  seconds.

Healthy rats weighing 74-156 g. were employed. All injections were made into a tail vein. Solutions of the alkaloids were prepared by dissolving weighed amounts in equimolecular quantities of hydrochloric acid.

For the first experiments rats were sacrificed at various intervals following injection of different alkaloids and their blood taken from the heart was subjected to prothrombin determination. Only in exceptional cases were two cardiac punctures made. The results are summarized in table 1. The values for retrorsine, pterophine and spartiodine were based on single animals, those for senecionine on the average of duplicates, those for monocrotaline were recorded as follows—with a dose of 330 mg. per kg. single animals were used for 3 separate intervals with 120 mg. per kg. 8 were sacrificed at the end of 4 hours, 5 at the end of 8 hours, and 12 at the end of 24 hours and with doses of 90 and 45 mg. per kg. all rats were bled twice by cardiac puncture. With scleratine single animals were sacrificed at the end of 48 and 72 hours on the

dose of 200 mg per kg, while the remaining figures in table 1 were the averages of 2 rats. These data clearly indicate that 5 out of 6 alkaloids definitely prolong the prothrombin time of rats when compared with the normal value of  $39.8 \pm 0.7$  seconds, and scleratino, the sixth member, has the same tendency although to a less degree. The amount of each substance required to induce hypoprothrombinemia is in the neighborhood of the median lethal dose ( $LD_{50}$ ) or greater, as judged by retrorsine and monocrotaline.

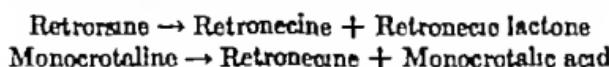
TABLE 1  
*Hypoprothrombinemic action of hepatotoxic alkaloids*

ALKALOID	SOLU- TION	NO. OF RATS	DOSE	PROTHROMBIN TIME (SECONDS)									
				Time after injection (hours)									
				2	4	6	8	12	24	28	30	32	48
Senecionine	1	16	70 mg per kg	48	52	74	162	1800+	1800+	1800+	1800+	1800+	1800+
Retrorsine	1	4	70	420	142	90		220		61	45		
		7	55	46	44	46	48	53					
Pterophine	1	7	90	55	42	55	70	62	90		570		
		7	60	47	47	38	54		181	1800+	360		
		8	40	110	47	38	36	40	48	46	46	46	
Spartioidine	1	3	100			70			540		420		
		1	80									1440	
Monocrotaline	5	3	330			60				160		360	
		25	120		43		61		1800+				
	1	4	90						56				49
		5	45									46	40
Scleratino	10	8	425			90		68		50	55		
		6	420		43		60	35					
	5	10	200		61		47	60		55		47	42
		10	130	45		51	57	55		52			

As pointed out in previous publications (4, 5, 6), extensive hemorrhage into the cords of liver cells, with or without much evidence of hepatic necrosis, was observed in those animals which had lived for 24 hours or longer following an intravenous injection of lethal doses of the hepatotoxic alkaloids. This hemorrhage may well be a direct result of hypoprothrombinemia, leading secondarily to necrosis of liver cells. Two other possibilities, however, cannot be ruled out. First, hypoprothrombinemia and necrosis of liver cells, together, may result in hemorrhage, and secondly, the hemorrhage is secondary to necrosis of liver cells. A direct deleterious effect of the alkaloids upon the liver cells is possible, since necrosis of single cells or groups of 2 or 3 cells was noted microscopically.

scopically without associated hemorrhage in some animals which were killed within 24 hours after the administration of the alkaloids.

The second series of experiments was undertaken to ascertain whether or not the cleavage products of these alkaloids possessed a similar action upon the rat's prothrombin time. Retrorsine and monocrotaline were employed as test examples. According to Manske (9) and Adams, Rogers, and Long (10), the 2 alkaloids can be hydrolyzed in the following manner:



It has already been pointed out previously that large doses of retronecine in mice injected intravenously caused immediate death but no liver injury (8).

TABLE 2

*Intravenous toxicity of retrorsine, monocrotaline, and their degradation products*

COMPOUND	SOLVENT	ANIMAL	NUMBER INJECTED	DOSE RANGE	LD <sub>50</sub> ± S.E.
Retrocine	%			mg per kg	mg per kg
	1	mice	40	62-90	58.8 ± 5.3
	1	rats	80	30-90	38.4 ± 2.8
Retronecine	5	mice	30	500-700	634.0 ± 26.0
	10	rats	15	900-1400	1311.0 ± 62.8
Retronecine lactone	5	mice	25	400-560	497.7 ± 18.9
	10	rats	25	400-700	589.8 ± 17.7
Monocrotaline	5	mice	63	200-400	261.3 ± 12.5
	5	rats	47	70-140	91.7 ± 3.8
Monocrotalic acid	5	mice	26	500-700	606.0 ± 16.3
	10	rats	26	500-700	580.8 ± 17.0

As a preliminary step to the present investigation median lethal doses of retronecine, monocrotalic acid, and retronecine lactone were determined in both mice and rats and compared with those of retrorsine and monocrotaline as shown in table 2. It is obvious that the degradation products are less toxic than the parent alkaloids. In contrast with retrorsine and monocrotaline, which produced delayed deaths, excessive doses of the degradation products either killed mice and rats promptly or not at all. There was no necrosis of the liver in those animals which survived the sublethal doses—they were sacrificed in 3-5 days.

Two rats received intravenously, retronecine in the dose of 1000 mg per kg, 20 others retronecine lactone in the dose of 500 mg per kg, and still 8 others monocrotalic acid in the dose of 500 mg per kg. They were bled twice or three times by cardiac puncture at the end of 12, 24, and 48 hours. All blood samples were tested for prothrombin time. The mean (geometric) of 20 determinations

was  $31.6 \pm 1.3$  seconds, as compared with  $39.8 \pm 0.7$  seconds for the control animals—a slight shortening of prothrombin time, in fact. In other words, the degradation products have no hypoprothrombinemic effect in rats. This is in harmony with the observation that the same substances do not cause liver necrosis. Thus, intact molecules of the natural alkaloids are required to induce hypoprothrombinemia and hepatic damages.

The next series of experiments was designed to detect any possible antagonism between vitamin K and hepatotoxic alkaloids. The latter were again represented by retrorsine and monocrotaline. Four groups of 18 rats were fed a diet containing 0.05% menadione. The average daily intake *per capita* was about 5 mg. After 10 days, the rats were injected intravenously with 2 dose levels each of retrorsine and monocrotaline, respectively, as shown in table 3. All surviving animals were sacrificed at various intervals, and their plasma pro-

TABLE 3  
*Action of retrorsine and monocrotaline in menadione treated rats*

ALKALOID	NUMBER OF RATS	DOSE	PROTHROMBIN TIME (SECONDS)					
			Time after injection (hours)					
			8	16	24	48	72	96
Retrorsine	16	70	43	53	49	46	44	38
	17	45	43	52	43	86	41	37
Monocrotaline	16	90	41	45	51	49	43	47
	18	70	48	39	34	36	37	36

thrombin times determined. The values in table 3 were averages of 2-3 animals. There is an apparent inhibition of the hypoprothrombinemic action, being particularly convincing with retrorsine in a dose of 70 mg per kg, and suggestive in other instances. Menadione does not completely detoxify these alkaloids, because 3 rats died with retrorsine, and 2, with monocrotaline, during the course of the experiments. Furthermore, slight to moderate central necrosis of the liver was observed microscopically in those animals which were sacrificed.

The last series of experiments consisted of 6 groups of rats on different diets, which are as follows: (a) regular colony ration, an entirely adequate diet which maintains optimal growth, (b) a simple but adequate purified diet containing Wesson's salts, (c) a highly purified low protein diet, (d) a diet consisting of 30% dried brewer's yeast and 70% diet (c), (e) a diet consisting of 15% Liver Extract, Lilly, and 85% diet (c), and (f) cracked corn. After the animals had received these diets for 2 weeks, senecionine in adequate doses was administered intravenously. The prothrombin time of the rats in each case did not show a significant difference from that of the control series.

## SUMMARY

1 Senecionine retrorsine pterophine spartiodine and monocrotaline in sufficient doses prolong the plasma prothrombin time of rats. Scleratine has a similar effect, but to a less degree.

2 Retronecine, retronecic lactone and monocrotalic acid—the degradation products of retrorsine and monocrotaline—do not cause liver necrosis in mice and rats, or hypoprothrombinemia in rats.

3 Feeding of a vitamin K preparation, menadione to rats appears to inhibit the hypoprothrombinemic action of retrorsine and monocrotaline.

4 Variation of the protein content of the diet and addition of brewer's yeast and liver extract have no appreciable effect upon the hypoprothrombinemic action of senecionine.

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## THIOURACIL LEVELS IN SERUM AND URINE

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Thiourea and thiouracil have been found to inhibit thyroid function (1-2). Because of the increasing employment of thiouracil in the treatment of thyrotoxicosis (3-10), it appeared desirable to study its concentration in the blood and excretion in the urine following its administration in therapeutic dosage to human subjects.

Several methods have been described for the determination of thiourea (11, 12, 13) and thiouracil (14), all based upon the development of a colored compound when Grote's reagent reacts with compounds containing a C=S group. The procedure described by Williams et al (14) proved unsatisfactory in our hands, that described by Chesley (13) for thiourea proved entirely satisfactory for thiouracil when modified slightly.

METHOD: Chesley's procedure (13) was adhered to with the following modifications. The pH of the serum filtrate and diluted urine was adjusted to 6.0 before adding the reagent and readings were made at 65 minutes in the Evelyn photoelectric colorimeter. According to Williams et al (14), maximum color development occurs at pH 8 and 15 minutes, but with the procedure employed by us color development was found to be maximal at pH 6 and 65 minutes. With filters ranging from 420 to 660, maximum absorption was obtained with filter 660. Higher filters were not available and the maximum may well be at a slightly higher wave length. However, filter 660 was considered satisfactory inasmuch as a linear curve was obtained when  $L = 2 - \log G$  was plotted against concentration and thiouracil added to serum and urine was recovered satisfactorily.

Control sera subjected to the same procedure yielded no visible color but gave readings slightly higher than those of aqueous blanks. These readings corresponded to thiouracil concentrations of 0.2-0.5 mg per 100 cc. A serum blank was therefore used with each determination, the galvanometer being set to give a reading of 100 with the serum blank (normal serum filtrate treated with Grote's reagent). With urine diluted 1:10, urine blanks gave readings identical with aqueous blanks and therefore the use of urine blanks was dispensed with.

The standard curves obtained by this procedure were quite uniform, but exact duplication was not always obtained. This is probably attributable to slight changes in the Grote reagent. Determinations on each set of unknowns were therefore made on the basis of a curve constructed from a series of standards run simultaneously.

A wide variety of compounds containing the characteristic sulfur linkage develop color with the Grote reagent (Williams et al (14)). From a practical standpoint, the most important of these are creatinine and glutathione. Creatinine added to serum in concentrations up to 10 mg per 100 cc and to urine in concentrations up to 150 mg per 100 cc gave no color with our procedure.

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No color was obtained with glutathione in serum in concentrations up to 10 mg per 100 cc and in urine in concentrations up to 50 mg per 100 cc.

**DISTRIBUTION IN THE BLOOD.** Thiourea is readily diffusible and has been used for the determination of body water (15). Determinations made upon the blood of subjects receiving thiouracil revealed essentially identical concentrations in serum and laked blood. This indicates that thiouracil is present within the erythrocytes in approximately the same concentration as in the serum.

Recovery of thiourea from serum ultrafiltrates (12) indicates that it is not firmly bound to serum proteins. However Williams et al (14) have recovered a portion of the serum thiouracil from precipitates of serum obtained with certain protein precipitants. This may be due either to adsorption of thiouracil by the denatured protein in vitro or to some form of combination of serum protein and thiouracil in vivo. In an attempt to investigate this matter serum

TABLE I  
Range of serum thiouracil values (mg %) following oral administration

TYPE OF ADMINISTRATION	HOURS											
	1	1	2	2	4	4	8	10	12	24		
0.2 gm single dose	0.0	7.0	0.70	0.70	~1.1							
0.2 gm 5 times (every two hours)			0	-1.10	7.1	5.0	0.1	7.1	3.2	0.1	3.2	3.1
1.0 gm. single dose	1.0	1.4	2.0	1.3-2.8	1.0-2.4	1.0	1.80	7.1	0			0
0.3 gm 5 times (every two hours)				1.6	2.2	2.3	2.7	3.0			0.8	0
0.4 gm 5 times (every two hours)				1.7	3.0	3.0	3.1	2.3			0.7	

samples from subjects receiving thiouracil were dialyzed (cellophane) against large volumes of 0.85% NaCl solution at 4°C for 18-20 hours. The serum samples contained 2.3-3.0 mg thiouracil per 100 cc. None was found in the serum after dialysis suggesting that thiouracil is present in the serum in a free state or that any combination with protein must be a very loose one.

**CONCENTRATION OF THE SERUM.** The concentration of thiouracil in the serum at varying intervals under different conditions of administration is indicated in table I. As has been reported by Williams et al (14) thiouracil can be detected in the serum in some instances as early as 30 minutes after oral administration of 0.2 gram. In a number of cases however none was demonstrable in the serum during a period of 2 hours after ingestion of 0.2 gram. The fact that appreciable amounts were found in the urine during this period indicates that the drug was absorbed. Following single oral doses of 0.3-1.0 gram thiouracil was detected in the serum in every instance during the first 2 hours.

When five doses of 0.2 gram were given at intervals of 2 hours during the day the level in the serum rose either promptly or gradually during the period of administration to a maximum of 1.4-2.3 mg per 100 cc (fig 1). Higher con-

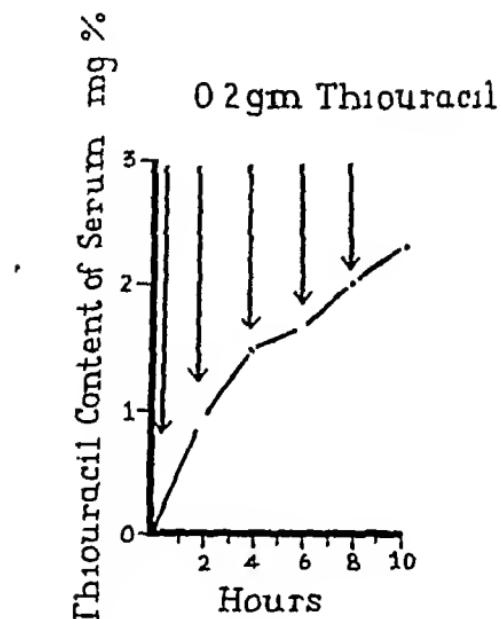


FIG 1 SERUM THIOURACIL LEVELS FOLLOWING ORAL ADMINISTRATION OF 1 GRAM THIOURACIL IN 5 DIVIDED DOSES OF 0.2 GRAM EACH

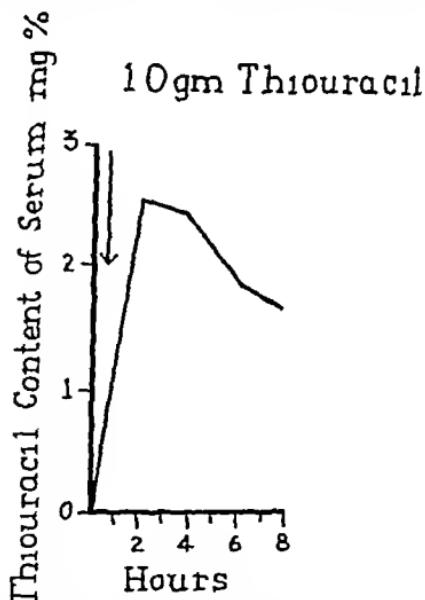


FIG 2 SERUM THIOURACIL LEVELS FOLLOWING ORAL ADMINISTRATION OF 1 GRAM THIOURACIL IN A SINGLE DOSE

concentrations were obtained with five doses of 0.3 or 0.4 gram, but the shape of the curves was essentially the same as with 0.2 gram doses. After administration of a single dose of 1.0 gram, the concentration in the serum reached a

maximum at 2 hours and fell subsequently (fig. 2). When 1.0 gram was given, either in five equally divided doses or as a single dose, during the day, none or only traces were present in the serum on the following morning. There was no apparent correlation between the concentration of thiouracil in the serum and the therapeutic response in patients with thyrotoxicosis when five doses of 0.2 gram were given at intervals of two hours during the day.

No information is available regarding the advisability of maintaining an adequate level in the serum during the entire 24 hour period, although this would appear to be desirable on theoretical grounds. When four doses of 0.1-0.2 gram and a final dose of 0.5-0.7 gram were given at intervals of two hours during the day concentrations of 0.7-0.9 mg per 100 cc were obtained in the serum on the following morning (12 hours after the last dose). When 1.0 gram of thiouracil was administered by rectum in suppositories in two equally divided doses, none was detected in the serum although about 10% was excreted in the urine during the 24 hour period.

**EXCRETION IN THE URINE.** After administration of 1.0 gram in five equally divided doses 22-57% is excreted in the urine during the first 24 hours and an additional 10-15% during the second 24 hours. After administration of 1.0 gram in a single dose we have observed excretion up to 64% in the first 24 hours and an additional 10-15% during the second 24 hours. In four cases receiving 1.5 and 2.0 grams in five equally divided doses the proportion excreted in the urine was considerably smaller ranging from 12.8 to 17.4% in the first 24 hours. Similar observations have been made by Williams (10). The excretion of 10-15% of the drug during the 24 hour period following the day of administration indicates its presence in the body fluids at this time but in concentrations too low to permit its demonstration in the serum by the method employed.

#### SUMMARY AND CONCLUSIONS

1. A modification of Chesley's procedure for determination of thiourea was found to be more satisfactory for thiouracil than methods described previously.

2. When administered orally thiouracil is absorbed rapidly and is also excreted rapidly in the urine. Absorption from the rectum is relatively poor and apparently inadequate for therapeutic purposes.

3. Thiouracil is present in the water of erythrocytes and plasma in approximately the same concentration. It can be removed from the serum by dialysis and is therefore either in a free state or only loosely bound to protein.

4. Curves of thiouracil concentration in the serum are described under different conditions of administration. A more constant level is maintained when small quantities are given at frequent intervals during the day than when a single large dose is administered. Unless a large dose is given in the evening the concentration falls during the night to practically imperceptible levels in the morning.

5. When 1.0 gram is given in divided doses, 22-55% is excreted in the urine during the first 24 hours and an additional 10-15% during the second 24 hours. With doses of 1.5 or 2.0 grams only 12.8-17.4% are recovered from the urine during the first 24 hours.

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# METABOLISM TOXICITY AND MANNER OF ACTION OF GOLD COMPOUNDS IN THE TREATMENT OF ARTHRITIS

## VII THE EFFECT OF VARIOUS GOLD COMPOUNDS ON THE OXYGEN CONSUMPTION OF RAT TISSUES

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Previous studies by Block Buchanan and Freyberg (1 2) have shown that when gold compounds are injected intramuscularly into white rats the gold is deposited in various tissues of the body primarily in the liver and kidney. The amount and site of deposition seem to depend on the physical properties of the compound studied. Also the severity of the histo-pathology in gold treated animals in general is proportional to the quantity of gold laid down in the tissue (3). In view of these findings it became of interest to us to know whether the respiration of these tissues, as measured by oxygen consumption, is influenced by the presence of gold and to determine whether a correlation exists between the concentration of these compounds in the tissue and the rate of respiration. If a relationship between deposition of gold and activity of the enzyme systems in these tissues could be found it might provide an insight into the mechanism of action of gold salts in the treatment of rheumatoid arthritis.

**PROCEDURE** Measurements of oxygen consumption were made with the usual type of constant volume Warburg manometer. The carbon dioxide produced was absorbed by 0.3 cc of 20 per cent potassium hydroxide solution contained in central wells on rolled filter paper. After introduction of the tissue slices the manometer flasks were immersed in a water-bath at 38° flushed with pure warmed oxygen (35°) for 10 minutes and shaken at a rate of 110 oscillations per minute. The suspending medium was a phosphate buffered physiological salt solution (pH 7.4) containing 0.2 per cent glucose as described by Krebs (4). The total volume of fluid in each flask was 3.0 cc.

The tissues were taken from healthy male and female white rats which had been fed a standard stock diet. The animals were killed by a blow on the head and the liver and kidney were quickly removed and washed free of blood. Slices of approximately equal size and thickness were made with a razor blade held against the lower side of a glass microscope slide. Readings of the manometers were made at 15 and 30 minute intervals. At the conclusion of the experiment the slices were removed from the flasks and dried for 24 to 48 hours at 90° and oxygen consumption was calculated per mg of dry weight of tissue.

The solutions to be tested were placed in the side arm at 10 times the final concentration and tipped into the main compartment containing the tissue after a control period of 45 minutes (5). In all cases the compounds were compared at equal molar concentrations.

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(M/500) with respect to gold. This represents the final dilution after tipping. Control studies in which the gold compound was omitted were made with every tissue slice.

**RESULTS** The gold-containing compounds used in these studies are listed in table 1. The in vitro effect of these gold compounds on the oxygen consumption of liver and kidney tissue slices is shown in Charts 1 and 2 respectively. The compounds fall into two groups, 1 those which caused an inhibition of respiration of the tissue slice, as evidenced by a decrease in oxygen consumption, and 2 those which have no effect on tissue respiration. In the first group are two very soluble, ionized compounds, gold chloride and gold sodium thiosulfate, and a totally different type of compound, colloidal gold sulfide. (There is some question as to whether the latter compound should be included in this group in view of its doubtful effect on respiration of liver tissue slice.) In the

TABLE 1  
*Gold compounds studied*

AuCl <sub>3</sub> HCl 3H <sub>2</sub> O GOLD CHLORIDE (50.0 PER CENT GOLD)	Na <sub>4</sub> Au (S <sub>2</sub> O <sub>3</sub> ) <sub>2</sub> GOLD SODIUM THIOSULFATE (37.4 PER CENT GOLD)	Au <sub>2</sub> S <sub>3</sub> COLLOIDAL GOLD SULFIDE (87.0 PER CENT GOLD)
$  \begin{array}{c}  \text{COONa} \\    \\  \text{H}-\text{C}-\text{S}-\text{Au} \\    \\  \text{CH}_3 \\    \\  \text{COONa}  \end{array}  $ Gold Sodium Thiomalate (50.0 per cent gold)	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{C}-\text{CH}_2 \\    \\  \text{Au}-\text{N} \\    \\  \text{C}-\text{CH}_2 \\    \\  \text{O}  \end{array}  $ $  \begin{array}{c}  \text{Na}^+ \\    \\  \text{4H}_2\text{O}  \end{array}  $ Sodium Succinimido Aurate (28.82 per cent gold)	$  \begin{array}{c}  \text{O} \\  \diagup \quad \diagdown \\  \text{HO}-\text{C}-\text{H} \quad \text{H}-\text{C}-\text{CH}_2\text{SAu} \\    \quad   \\  \text{H}-\text{C}-\text{OH} \quad \text{HO}-\text{C}-\text{H} \\    \quad   \\  \text{H} \quad \text{C} \\    \\  \text{OH}  \end{array}  $ Gold Thioglucose (50.0 per cent gold)

Second group are three compounds in which the gold is non-ionized form as part of complex organic molecules

The inhibition of tissue respiration by gold chloride and gold sodium thiosulfate probably is due to the presence of gold ions in the reaction medium, because differences in hydrogen ion concentration were controlled by adjusting the reaction of the highly ionized gold solutions to pH 7.0 with sodium hydroxide and by the use of the phosphate buffer in the Ringer solution. Further evidence that the decrease in oxygen consumption may be attributed to gold ions was furnished by normal control studies with sodium thiosulfate, sodium thiomalate, succinimide, and thioglucose. An explanation of the inhibitory effect of colloidal gold sulfide is difficult since presumably no ionic gold is present. However, it is possible that not all the gold is in colloidal form and some gold ions are present to cause inhibition.

Experiments were undertaken to determine the effect of various concentrations of gold ions. Solutions of gold chloride varying in concentration from M/250 to M/500 to M/1000 produced the same relative degree of inhibition.

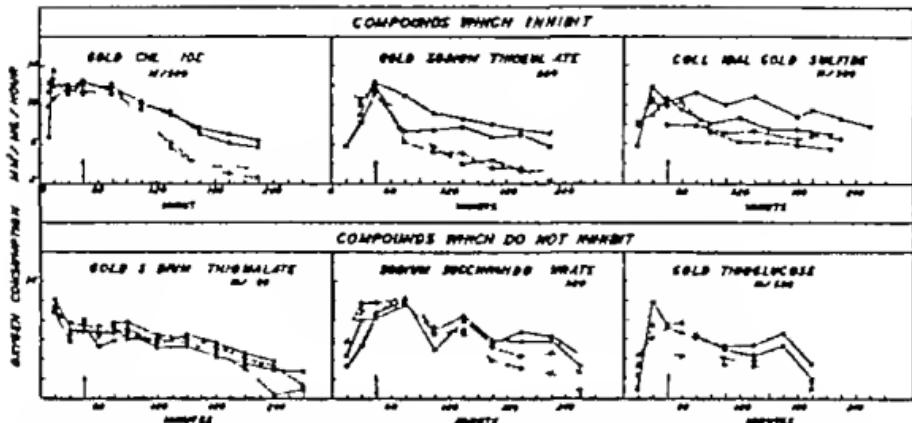


CHART 1 THE EFFECT OF VARIOUS GOLD-CONTAINING COMPOUNDS UPON THE OXYGEN CONSUMPTION OF RAT LIVER TISSUE SLICES

The vertical arrows indicate the time of addition of gold. Control studies are represented by solid lines; experimental studies by broken lines. Different symbols represent determinations on liver samples from different animals.

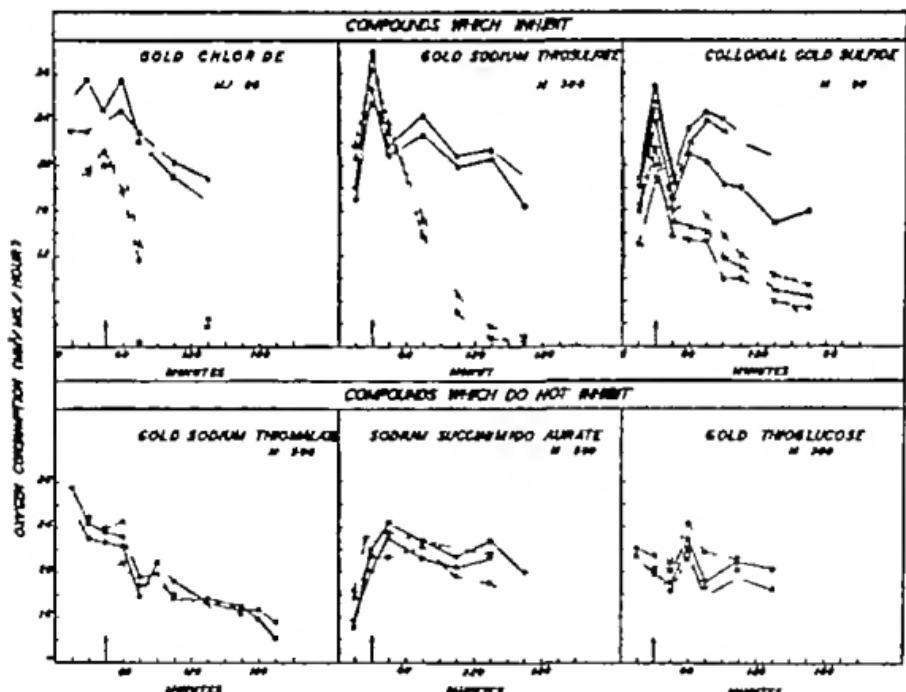


CHART 2 THE EFFECT OF VARIOUS GOLD-CONTAINING COMPOUNDS UPON THE OXYGEN CONSUMPTION OF RAT KIDNEY TISSUE SLICES

The vertical arrows indicate the time of addition of gold. Control studies are represented by solid lines; experimental studies by broken lines. Different symbols represent determinations on kidney samples from different animals.

Slightly less inhibition was obtained with M/5000 gold chloride and no observable effect with M/50,000.

From Charts 1 and 2 it is obvious that the compounds of Group 1, that is, soluble ionized compounds (gold chloride and gold sodium thiosulfate), in equal concentrations have a much more pronounced effect on the oxygen consumption of kidney tissue than of liver tissue. This difference is interesting because it has been noted previously, Block et al. (1), that the soluble gold-containing compounds are deposited in the body to a greater extent in the kidney than in the liver. Although the soluble organic gold compounds (sodium succinimidoaurate, gold sodium thiomalate, and gold thioglucose) also are excreted by and deposited primarily in the kidney, they had no effect on the oxygen consumption of kidney slices, presumably because of the absence of ionized gold.

It is interesting to note that the compounds which have no effect on oxygen consumption of tissue slices *in vitro*, sodium succinimido-aurate, gold sodium thiomalate, and gold thioglucose, are the compounds which have been found to produce the least toxic reactions in the treatment of rheumatoid arthritis in human patients.

#### SUMMARY

1. The *in vitro* effect of various gold-containing compounds upon the oxygen consumption of liver and kidney slices from healthy white rats was determined.

2. The oxygen consumption was inhibited by the inorganic, ionizable compounds, gold chloride and gold sodium thiosulfate, and to a lesser degree by colloidal gold sulfide. The organic, non ionizable compounds, sodium succinimido-aurate, gold sodium thiomalate, and gold thioglucose, did not cause any inhibition. At equal molar concentrations of gold the degree of inhibition was greater for kidney than for liver slices.

3. Solutions of gold chloride varying in ionic concentration from M/250 to M/5000 produced the same relative degree of inhibition on the oxygen consumption of liver and kidney slices.

4. The significance of these findings in relation to earlier studies on the distribution of gold in tissues is discussed briefly.

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# RENAL CLEARANCE OF SULFAMERAZINE, SULFADIAZINE, SULFATHIAZOLE, AND SULFAPYRIDINE IN MAN<sup>1</sup>

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The manner by which excretion of the sulfonamides is accomplished by the kidney is of major importance among the factors governing the behavior of these drugs in the body. The different yet characteristic concentrations obtained in blood plasma when various sulfonamides are administered at comparable dosages depend to a considerable extent upon the rate at which they are excreted by the kidney. This in turn will be determined by the predominance of glomerular or tubular excretory pathways and upon the reabsorptive activity of the tubules. Toxic effects upon the kidney of poorly soluble sulfonamide compounds are also related to the extent that their concentration increases in the urine as it passes through the tubules. The concentration rise will be determined by the amount filtered through the glomeruli and upon the proportion secreted or reabsorbed in the tubules.

Investigation of the renal excretion of sulfanilamide, sulfapyridine and certain other compounds has been made by Marshall and his collaborators (1, 2, 3) and by others. Recently Loomis, Hubbard and Koeps (4) have compared clearances of sulfanilamide and acetyl sulfanilamide with clearance of inulin by rabbits. A comprehensive study of the relation of structure to excretion and other pharmacodynamic properties of these compounds in animals has been made by Fisher, Troast, Waterhouse and Shannon (5). Beyer, Potors, Patch and Russo (6) have investigated the influence of diuretics and alkali administration upon clearances of several sulfapyrimidines and of sulfathiazole in the dog.

The excretion by man of certain of the sulfonamides has been investigated (7-10). However the unavailability for excretion by the kidney of varying amounts of these compounds because of combination between plasma protein and the drug was overlooked by earlier studies. This factor together with the pathway of excretion by the kidney whether by the glomeruli or tubules has received consideration only in the recent reports of Loomis, Koeps and Hubbard (11) who measured simultaneously the clearances of sulfanilamide, acetyl sulfanilamide and inulin and of Earle (10) who compared clearances of sulfamerazine, acetyl sulfamerazine and sulfadiazine with the clearance of mannitol.

It was the purpose of the present study to compare several commonly used sulfonamides, sulfapyridine, sulfathiazole, sulfadiazine and sulfamerazine with

<sup>1</sup> Aided by grants from Sharp and Dohme Inc., Glenolden, Pa., and the Fund for Research in Infectious Diseases, University of Pennsylvania, Philadelphia.

respect to the manner of their excretion by the human kidney. For this purpose, the clearance of the sulfonamides and of inulin were measured simultaneously. The protein-bound sulfonamide of plasma was measured by ultrafiltration and the clearances were corrected for this factor.

**EXPERIMENTAL** Young or middle aged male patients, awaiting discharge from the Medical Department following recovery from various pathological states (mainly pneumonia), served as subjects. Those that received sulfapyridine, sulfathiazole, or sulfamerazine showed no evidence of gastrointestinal, heart, or kidney disease, but the group employed for the investigation of sulfamerazine could not be as rigorously selected and several presented evidence of impaired kidney function.

Each subject received 3 gm. of sulfonamide as the sodium salt, together with 5 to 10 gm. of inulin by vein, following ingestion during the preceding 2 hours of sufficient water to induce diuresis. Clearances were done 2 to 3 hours after a light breakfast, quantitative collections of urine being made during 30 to 60 minute time periods. Other methods employed were those listed in a previous report (9). Plasma inulin concentrations used in calculating clearances were obtained by interpolation based usually on three or more plasma samples. The patients were not catheterized. In most experiments large urine flows were established in order to diminish error due to incomplete collection of urine. In general, the procedure employed in measuring inulin clearance followed that proposed by Alving and Miller (12). Inulin was determined by the method of Alving, Rubin, and Miller (13), observing precautions described by Alving, Flox, Pitesky and Miller (14). In the experiments dealing with sulfamerazine the method of Hubbard and Loomis (15) was substituted\*. Inulin purified by the method of Smith (16) was used in most experiments. All inulin used was free of pyrogenic action.

Calculations of clearances were based upon that portion of plasma sulfonamide immediately available to the action of the kidney, namely, the unbound portion, as determined by ultrafiltration. The method of Lavietes (20) was employed for ultrafiltration. Correction of clearances for the protein bound sulfapyridine, sulfadiazine, and sulfathiazole of plasma was based on averaged results of ultrafiltration experiments using plasma from similar sources and containing similar concentrations of sulfonamides (17), while those of sulfamerazine were based upon actual ultrafiltration of the plasma obtained during the clearance study. Ultrafilterable fractions of sulfapyridine, sulfathiazole, sulfadiazine, and sulfamerazine were found to average 0.60, 0.45, 0.75, and 0.41 respectively at 37.5°C., at the average plasma concentrations shown in tables 1 to 4. Clearances of the sulfonamides were calculated from the usual formula  $UV/P$  where  $U$  is the concentration in urine,  $V$  the volume of urine per minute and  $P$  the concentration in plasma water.

**RESULTS** Observed clearances of the four compounds were in each instance lower than the simultaneously determined inulin clearance. In descending order, these were for sulfathiazole 39.3, sulfadiazine 22.6, sulfapyridine 16.5, and sulfamerazine 8.3 per cent (in the group with normal renal function) of the inulin clearance. However, the four sulfonamides studied were combined to a varying extent with plasma protein and the fraction so combined was rendered unavailable for filtration through the glomerular membrane. When corrections were made for the protein-bound portion, the ratio of the clearances of sulfadiazine and sulfapyridine to the inulin clearances (excretion ratios) were 0.31 and 0.28 respectively (tables 1 and 3). The excretion ratio of sulfamerazine

\* Hydrolysis for 16 minutes instead of 8 proved advantageous in the method of Hubbard and Loomis. This modification gave a more stable color of greater density than that obtained by following the original directions.

TABLE 1  
*Sulfapyridine and Insulin clearances*

PATIENT NO.	PLASMA SULFAPYRIDINE		URINE		CLEARANCES		ST INSULIN	AC. ST INSULIN	
	Free	Acet.	Volume	Sulfapyridine		Free	Acet. <sup>†</sup>		
				Free	Acet.				
	mg. per 100 cc.	cc./min.	mg. per 100 cc.			cc. per 1.73 sq. m.			
15	5.0	1.4	8.8	10.0	3.0	27.9	17.4	88.1	
	5.5	0.9	3.0	22.0	3.0	24.9	12.5	101.5	
15	5.1	1.0	2.4	50.0	10.0	40.5	30.1	162.3	
	4.4	0.8	2.8	48.0	11.0	61.4	48.0	209.5	
17	4.8	1.1	12.4	9.8	2.8	49.5	30.0	219.8	
18	5.3	1.4	2.9	26.0	7.8	14.0	11.5	75.9	
	5.7	1.0	7.3	10.0	2.5	15.5	13.5	77.0	
19	8.5	0.8	12.0	12.5	1.0	31.1	15.9	68.8	
	8.4	0.5	7.0	18.0	3.0	24.7	45.3	79.5	
Mean.						33.5	20.0	120.2	
S.D.						±17.2	±15.0	±58.9	
								±0.03	
								±0.14	

\* Clearances of both free and acetylated sulfonamides are calculated on the basis of concentrations in the ultrafiltrate except where indicated

† Not corrected for protein bound fraction.

Means are calculated on the basis of the total number of experiments

TABLE 2  
*Sulfathiazole and Insulin clearances*

PATIENT NO.	PLASMA SULFATHIAZOLE		URINE		CLEARANCES		ST INSULIN	AC. ST INSULIN	
	Free	Acet.	Volume	Sulfathiazole		Free	Acet.		
				Free	Acet.				
	mg. per 100 cc.	cc./min.	mg. per 100 cc.			cc. per 1.73 sq. m.			
0	8.8	1.2	2.0	175.0	30.0	82.5	223.0	88.6	
	9.0	1.0	8.8	23.8	2.2	58.9	88.2	84.4	
	8.7	0.3	11.5	25.0	3.0	72.2	515.0	94.7	
10	7.0	0.8	18.1	25.8	5.2	119.8	505.1	199.3	
	8.5	1.7	17.6	22.8	3.5	123.8	158.6	150.9	
11	11.8	1.0	12.5	34.0	5.0	79.1	338.8	109.0	
12	8.1	1.8	23.0	32.0	5.0	191.3	347.2	196.8	
	7.4	1.4	27.5	23.0	3.0	181.8	263.9	188.1	
13	10.2	8.1	2.3	210.0	50.0	108.9	183.4	117.2	
	8.5	1.3	24.1	20.5	3.5	133.8	310.0	181.3	
	7.5	1.9	12.8	25.0	3.3	97.6	111.9	109.7	
14	9.0	1.3	4.4	110.0	12.0	138.9	224.1	182.8	
	8.1	1.8	3.5	134.0	21.0	153.8	321.7	187.6	
Mean.						118.7	276.1	135.8	
S.D.						±41.1	±133.1	±41.2	
								±0.15	
								±1.18	

See footnote below table 1

was appreciably lower, 0.20 (table 4). Sulfathiazole (table 2) differed from the other compounds studied in that its corrected clearance approximated the inulin clearance, with an excretion ratio of 0.87. Individual excretion ratios extended from 0.60 to 1.16. The difference between the means of sulfathiazole and inulin clearances was not significant when Fisher's *t* test (18) was applied. However, the group examined was small, and it is possible that examination of a larger population would reveal some difference.

The contrast between the behavior of sulfathiazole compared with that of sulfapyridine, sulfadiazine, and sulfamerazine is shown in figure 1, where the relationship between the quantity of each compound appearing in the urine is

TABLE 3  
*Sulfadiazine and Inulin clearances\**

PATIENT NO	PLASMA SULFADIAZINE		URINE		CLEARANCES			SD INULIN	AC. SD INULIN		
	Free	Acet.	Volume	Sulfadiazine		Sulfadiazine					
				Free	Acet.	Free	Acet.				
	mg per 100 cc.		cc/min	mg per 100 cc.		cc per 1.73 sq m					
1	12.5	1.0	5.1	60.0	12.0	32.8	91.4	89.5	0.37		
2	11.3	0.8	15.8	16.4	3.8	27.6	103.7	109.5	0.25		
3	13.4	0.9	14.2	20.0	1.8	28.5	42.6	102.0	0.28		
	12.3	1.1	13.0	20.8	1.7	29.6	29.3	136.7	0.22		
4	18.2	1.1	19.6	27.0	3.0	33.3	71.8	89.0	0.37		
	16.5	0.8	21.3	22.6	4.0	33.6	155.6	107.9	1.47		
5	13.4	0.8	17.1	22.5	2.5	28.6	61.8	97.5	0.29		
	11.6	0.8	15.3	21.5	2.5	26.6	55.3	89.6	0.30		
6	10.8	0.5	20.1	22.0	2.0	45.8	102.4	161.6	0.28		
	10.3	0.7	17.4	20.4	2.4	31.6	73.1	145.9	0.22		
7	15.6	0.9	11.5	48.0	6.0	62.0	147.5	123.2	0.50		
	14.5	0.4	12.1	32.0	4.0	46.5	235.2	89.6	0.52		
8	16.0	1.5	12.6	28.0	3.0	19.7	26.2	106.7	0.19		
	14.4	1.6	18.1	19.0	2.0	21.9	22.5	126.3	0.17		
Mean						33.4	87.2	112.5	0.31		
SD						±12.5	±59.8	±23.1	±0.11		
									±0.64		

\* See footnote below table 1

plotted against the quantity filtered through the glomeruli. The quantity filtered is calculated from the inulin clearance and the sulfonamide concentration in the ultrafiltrate. Substances excreted only by filtration without participation of the tubules yield figures that fall along the broken line. Sulfathiazole clearly does so, and it therefore belongs to the group of substances that are filtered by the glomeruli but neither secreted nor removed during passage through the tubules to any considerable extent and its excretion is directly proportional to the concentration in the ultrafiltrate. Substances appearing below the diagonal, those with lower output in the urine than in the glomerular filtrate, have been reabsorbed by the tubules to a greater or lesser extent. Excretion of these also increases with rise in concentration of drug in ultrafiltrate, but not as much nor is there direct proportionality. It is apparent that sulfapyridine, sulfamerazine, and sulfadiazine are secreted by the tubules to some extent.

diasine and sulfamerazine belong in this category. The fraction of these three compounds reabsorbed by the tubules is 0.72, 0.69, and 0.80 respectively.

TABLE 4  
*Sulfamerazine and Inulin clearances*

PATIENT NO.	PLASMA SULFAMERAZINE		URINE		CLEARANCES		SEM INULIN
	Free	Ultralfiltrate	Volume	Sulfamerazine Free	Sulfamerazine Free	Inulin	
Subjects with normal kidney function							
	mg per 100 cc.	cc./min.	mg per 100 cc.	cc. per 1.73 sq. m.			
20	14.4	6.0	11.3	8.7	17.0	180.0	0.09
	13.4	6.6	4.6	16.7	13.0	91.2	0.14
	11.6	4.9	1.4	50.3	15.1	153.0	0.10
21	16.6	6.1	3.2	78.6	35.7	156.0	0.23
	8.9	3.3	1.6	64.7	27.7	106.0	0.26
22	25.1	11.3	9.0	20.7	15.1	73.9	0.20
	23.0	10.4	7.7	43.3	29.8	106.7	0.28
25	10.4	4.9	6.9	14.2	17.9	144.0	0.12
	0.7	4.6	2.0	22.0	12.4	94.6	0.18
27	24.1	10.8	10.1	20.7	24.5	101.4	0.24
	20.8	0.6	11.3	28.4	27.7	94.0	0.29
	20.4	9.2	10.6	23.9	24.8	88.2	0.28
29	12.9	4.5	3.9	22.8	17.6	92.3	0.10
30	23.1	9.6	8.2	16.9	15.7	85.6	0.18
Mean					21.0	112.4	0.20
S.D.					±7.2	±33.3	±.07
Subjects with impaired kidney function							
	mg per 100 cc.	cc./min.	mg per 100 cc.	cc. per 1.73 sq. m.			
23	14.8	6.7	2.8	33.7	11.4	69.5	0.18
	14.3	6.4	1.3	70.5	13.6	62.5	0.22
	13.5	6.1	0.8	83.4	11.2	47.0	0.24
24	14.6	5.0	6.2	8.3	9.2	52.6	0.18
	13.6	4.6	6.4	9.1	11.4	59.2	0.19
	12.3	4.2	6.3	10.2	11.6	60.8	0.19
26	20.3	10.0	3.6	32.3	11.2	40.1	0.23
	19.7	9.7	2.0	77.4	16.6	61.9	0.30
28	17.3	4.7	5.7	9.5	9.8	78.9	0.12
	16.1	4.4	1.6	23.7	7.2	62.8	0.11
Mean					11.2	58.5	0.20
S.D.					±2.3	±11.3	±.06

See footnote below table 1

Moderate impairment of kidney function<sup>3</sup> existed in four of the subjects employed for the study of sulfamerazine clearance. Clearances of sulfamerazine

<sup>3</sup> Patient No. 23 had mild rheumatic heart disease and No. 24 luetic heart disease. No. 26 showed renal colic and hematuria accompanying marked crystalluria that cleared up 7 days before the clearance was measured. No explanation could be found for the lowered inulin clearance in No. 28.

and of inulin were diminished to the same extent in these individuals as is shown by the identical excretion ratios of the groups with normal and subnormal clearances.

The acetyl derivatives showed higher clearances than the free forms (tables 1, 2, 3). When correction is made for the protein-bound fraction, the clearances of acetyl sulfathiazole equal or often exceed the inulin clearance by a considerable margin. Thus, excretion of acetyl sulfathiazole appears to be partially and perhaps sporadically by way of the tubules. This may be true also in certain individuals who have received sulfadiazine. Despite the high clearances, only small concentrations of the acetylated compounds appear in the urine. The high clearances are due to the low concentrations of acetyl compound in plasma.

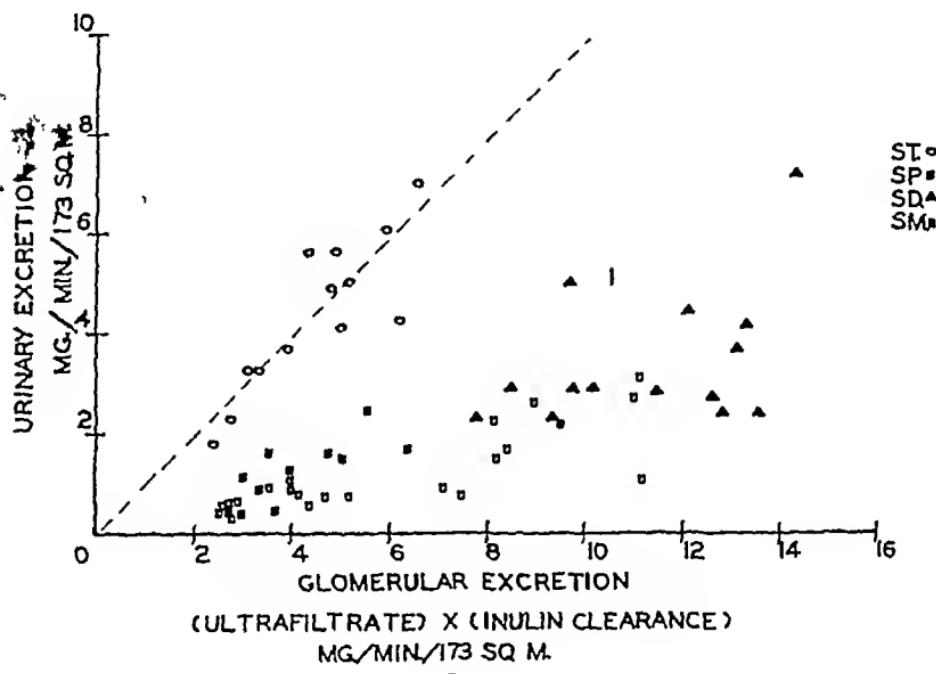


Fig. 1

available for excretion, since the proportion bound by plasma protein is higher than that of the free sulfonamide.

Clearance of acetyl sulfamerazine could not be evaluated as accurately as that of the other sulfonamides because of technical difficulties in connection with its determination. Satisfactory data were obtained to permit calculation of corrected  $\lambda$  values for four patients. In three (Nos. 22, 24, 27) the clearance of acetyl sulfamerazine ranged from 1.6 to 6.4 times the inulin clearance, in the fourth it was 0.47 of the latter.<sup>4</sup> Concentrations in plasma in these experiments

<sup>4</sup>The low acetyl sulfamerazine clearance of this subject (No. 28) was mainly caused by an aberrant value (0.38) for bound drug. If the average value found for other subjects is substituted, the clearance rises to 1.13 times the inulin clearance.

were 0.8 to 8.0 mg per 100 cc. Only 30 per cent of the acetyl sulfamerazine was ultrafilterable.

Urine volume was without direct effect on clearance of sulfathiazole or sulfamerazine. In this our results are in agreement with those of Lucas and Murchell (19) and Loomis Koepf and Hubbard (11) who examined sulfanilamide. Beyer Peters Patch and Russo (6) found some increase in output of sulfonamides by dogs when urine flow increased from very low values and Earle (10) found increased sulfamerazine clearance when diuresis was instituted in dogs. The effect of urine volume on sulfadiazine and sulfapyridine clearance cannot be evaluated in our experiments since they failed to include a sufficient range of urine volumes.

Urea clearances decreased during a 2 day period of oral administration of sulfathiazole (2 subjects) and of sulfapyridine (2 subjects) when these drugs were administered at the rate of 1 gram every 4 hours. The change appeared to be greater following sulfapyridine. Return to original values followed when administration was discontinued. Diminution of urea clearance was observed in numerous patients treated for pneumonia by means of sulfonamide compounds (8, 9). The similar response obtained in normal individuals indicates that a manifestation of the depressant action of one substance on excretion of others by kidney was not the result of the complicating effects of disease on kidney function in the individuals previously studied.

**DISCUSSION** Our findings for men agree with those of Fisher Troast Waterhouse, and Shannon (5) who investigated sulfonamide clearances of dogs. They found that sulfathiazole clearance when corrected for bound drug was equal to the glomerular filtration rate measured by creatinine clearance. Sulfadiazine, sulfamerazine and sulfamethazine were extensively reabsorbed by the tubules.

Comparison of the results of Earle (10) with those presented in this paper is best made on the basis of the excretion ratio (clearance of unbound sulfamerazine divided by the glomerular filtration rate). In his 4 subjects this averaged 0.15 and the average of 24 measurements in our 7 subjects was 0.20. The difference is not statistically significant. Excretion ratios for sulfadiazine were in close agreement with those reported by Earle. Earle found that acetyl sulfamerazine is secreted by the tubules and our observations lead to a similar conclusion.

Some question can be raised as to the significance of the clearance measurement when applied to sulfonamides that may undergo alteration in the body and be excreted in several forms not distinguished by the analytical method employed. Scudi and Jelmek (21) found several derivatives of sulfapyridine in urine in large quantity but the other three compounds yielded smaller amounts. Their significance in man has not been evaluated. Despite his possible objection it is of interest to provide a comparison of the four clinically important sulfonamide compounds studied by the means at present available for measuring the rate of their excretion. It is well to recognize that while the clearances measured are predominantly those of the sulfonamides themselves derivatives retaining the aromatic amino group may be contributing. Such derivatives could be expected to show differences in the renal pathway by which

they are excreted. If their formation was sporadic and variable, this would be reflected in increased variations in the sulfonamide clearance as compared with the simultaneously measured inulin clearance. Actually, only sulfadiazine clearances show appreciably greater variability than the inulin clearance. Clearances of other drugs showed little evidence of such variation, which suggests either that (1) formation of such derivatives was comparatively insignificant, (2) they were handled by the kidney in the same manner as the sulfonamide itself, (3) under the conditions of these experiments they formed a constant proportion of the total excretion with little individual variation.

#### SUMMARY

Renal clearances of sulfamerazine, sulfadiazine, sulfapyridine, sulfathiazole, acetyl sulfamerazine, acetyl sulfadiazine, and acetyl sulfathiazole have been measured in men and compared with the simultaneously determined clearance of inulin. Results were corrected for sulfonamide unavailable for excretion due to combination with plasma protein.

Sulfathiazole differed from the other three sulfonamides in showing a mean clearance averaging 87 per cent of the inulin clearance. Thus, reabsorption by the tubules was of minor significance.

The clearance of sulfamerazine averaged 20 per cent, sulfapyridine 28 per cent, and sulfadiazine 31 per cent of the inulin clearance, indicating that these compounds are extensively reabsorbed by the tubules.

Acetylated sulfonamides have higher clearances than the free forms. Acetyl sulfamerazine and acetyl sulfathiazole appear to be excreted by a combination of glomerular filtration and tubular secretion. Acetyl sulfadiazine shows a similar behavior, but the filtration-reabsorption pathway seems to be the predominant one.

Four subjects with moderately impaired renal function showed the same ratio of sulfamerazine to inulin clearance as those with normal renal function.

The temporary diminution in urea clearance previously observed during therapeutic administration of sulfonamides occurs also in normal individuals receiving comparable amounts of these drugs.

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## STUDIES ON ANTIMALARIAL DRUGS

### THE DISTRIBUTION OF ATABRINE IN THE TISSUES OF THE FOWL AND THE RABBIT<sup>1</sup>

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The work reported here was undertaken with a view to studying the accumulation and persistence of atabrine in the fowl after single and repeated intravenous injections of the drug. Although the metabolism of atabrine has been studied in several mammalian species (1-5) the results are not easily correlated due to variations in dosage schedules and routes of administration, and in some cases, to inadequate analytical methods. Hence, we have also studied the atabrine metabolism in the rabbit so that the behavior of the drug in an avian and a mammalian species could be compared.

**EXPERIMENTAL** The rate of disappearance of atabrine in chickens and rabbits was studied in animals receiving 5 mgm./kgm. of atabrine dihydrochloride intravenously (in the wing vein of the chicken and the marginal ear-vein of the rabbit) and sacrificed at intervals of 10 minutes to four days. The rate of accumulation was studied in animals which received 1, 5, 10 or 21 (rabbit only) daily injections, and which were sacrificed one hour after the last dose. *In vitro* studies were done to determine whether atabrine (like quinine) could be degraded by certain tissue extracts (6, 7). Experiments were carried out on three pregnant rabbits to determine the extent of placental transmission of atabrine. All doses and tissue concentration data are expressed as atabrine dihydrochloride.

Atabrine in the tissues was extracted by a method previously described (3). In the case of the rabbit, the tissue digests were extracted twice with ether, a procedure which gives 85% recovery in the liver and 90% recovery in the lung and kidney. A correction factor was therefore applied to these tissues. In the chicken, the tissue digests were extracted three times with ether which obviated the necessity of applying a correction factor. Blood atabrine was determined by the method of Brodie and Udenfriend (8).

In the lung, liver and kidney it was felt that the distribution within the organ might be uneven, therefore the total organ or a large aliquot was digested with the dilute HCl and blended with water in a Waring Blender. Aliquots representing usually one gram of tissue were withdrawn for analysis. All analyses were done in duplicate.

In all cases, the final sulfuric acid extract was diluted with an equal volume of alcohol and 1 cc. of concentrated NH<sub>4</sub>OH added for each 50 cc. of solution.

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<sup>2</sup> John J. Abel Fellow in Pharmacology.

Readings were made with the Coleman Model 12 A Electronic Photofluorometer using a standard solution containing 1 microgram/cc and subjected to the extraction procedure. Readings of less than 2 divisions on the instrument (equivalent to 0.02 microgram/cc) were recorded as 'trace'. Tissues from animals which had never received atabrine read between zero and 0.5 division.

**RESULTS AND DISCUSSION** The rabbits and chickens receiving repeated daily doses of 5 mgm/kg of atabrine showed no toxic effects and lost no weight. A limited number of acute toxicity experiments on rabbits served to confirm the findings of Hecht (9) and of Dawson Gingrich and Hollar (10) that the intravenous toxicity was in the neighborhood of 10 mgm/kgm. For acute toxicity experiments in chickens white leghorns weighing approximately 200 grams were injected with 1% solutions of atabrine dihydrochloride at the rate of 1 cc per minute. The LD<sub>50</sub> was found to be 35 mgm/kgm under these conditions.

TABLE 1

Atabrine content in mgm/kg of tissues of rabbits at various intervals after the intravenous injection of 5 mgm/kg of atabrine dihydrochloride

SAMPLE	TIME AFTER INJECTION							
	10 min.	1 hour	24 hours	48 hours	96 hours			
Lung	126	189	67	78	84	42	29	16
Kidney	61	100	69	63	21	21	30	16
Spleen	21	84	40	53	27	25	30	16
Adrenal	23	22	37	27	23	14	9	6
Liver	25	23	20	20	8	22	20	12
Heart	31	28	15	14	5.5	4.7	2.6	2.6
Brain	5.4	3.7	3.5	3.0	1.0	0.9	trace	0.6
Muscle	trace	1.4	2.8	1.6	0.8	0.9	0.1	0.5
Bile	0	0			1.7	1.5	2	trace
							trace	trace
								0

Cortex = 24 medulla = 15

**ACCUMULATION AND PERSISTENCE IN THE TISSUES OF THE RABBIT AND FOWL.** Data on the tissue distribution of atabrine in the rabbit are summarized in tables 1 and 2 and in the fowl in tables 3 and 4. The most striking difference between the two species is the more rapid elimination of atabrine in the fowl. Forty-eight hours after the intravenous injection of 5 mgm/kg of atabrine only very small amounts of the drug were found in the tissues of the fowl while all tissues of the rabbit except for muscle and brain contained quite high concentrations. Even after 96 hours, the concentrations in the rabbit were higher than those found in the bird after 48 hours. Further evidence of the more rapid metabolism of atabrine in the chicken is seen by comparing the extent of accumulation of repeated doses in the two species (tables 2 and 4). After similar dosage the concentrations of atabrine found in the tissues of the fowl with the exception of the liver were much lower than those in the rabbit.

In a single experiment a rabbit was given 10 mgm/kg. intravenously as a 0.5% solution. With this higher dose there was increased storage in the main depots i.e., the lungs, kidney, spleen, adrenal and liver (table 2).

TABLE 2

Atabrine in tissues of rabbits receiving atabrine dihydrochloride intravenously and sacrificed one hour after 1, 5, 10 and 21 daily injections

SAMPLE	NUMBER OF DAILY INJECTIONS								
	1		5		10		21		1
	Daily dose (mgm / Kg)								
	5	5	5	5	5	5	5	10	
	mgm / kg	mgm / kg	mgm / kg	mgm / kg	mgm / kg	mgm / kg	mgm / organ	mgm / kg	
Lung	67	78	294	222		360	147	23	
Kidney	69	63	140	105	173	182	182	96	
Spleen	40	53	101	65	133	106	413	33	
Adrenal	37	27	56	43	68	64	120	97	
Liver	20	20	52	47	80	70	144	45	
Heart	15	14	42	48	44	52	129	38	
Brain	3.5	3.0	8.4	4.5	5.0	7.0	12.5	4.5	
Muscle	2.8	1.6	3.0	5.1	6.5	5.0	5.3	2.0	
Bile			8.0	8.6	3.5	9.0	15	3.5	

TABLE 3

Atabrine content in mgm / kg of tissues of chickens at various intervals after the intravenous injection of 5 mgm / kg of atabrine dihydrochloride

SAMPLE	TIME AFTER INJECTION				
	10 min	1 hour	24 hours	48 hours	
Lung	22	20	11	12	4.3
Kidney	30	38	23	42	7.5
Spleen	55	44	62	43	8.0
Adrenal	32	33		53	14.8
Liver	62	94	61	38	9.3
Heart	14	12.5	8.6	11	1.5
Brain	4.0	5.0	5	4.5	1.5
Muscle	3.0	3.5	2.8	2.0	trace
Bile	5.3	4.0	6.2	16	2.0
Pancreas	22	22	26	20	4.4
Bone marrow	6.5	9.5	11	10	2.0
Testis	2.2	2.5	2.6	7.0	3.0
Blood plasma	0.43	0.62		0.09	0.7

Total atabrine in mgm / organ

Lung	0.18	0.19		0.09	0.04	0.03	0.02	0.01
Kidney	0.29	0.33		0.32	0.06	0.07	0.03	0.02
Liver	1.64	2.21		1.40	0.34	0.28	0.05	0.06

In the rabbit, the greatest tissue concentration of atabrine was found in the lung, though if the organ weights are taken into consideration, the greatest total amount of atabrine was normally found in the liver. An exceptionally

high atabrine content was obtained in the lung of the pregnant rabbit shown in table 5. This animal had convulsions and respiratory embarrassment shortly after the injection. In another rabbit which died in convulsions immediately after the intravenous injection of 15 mgm./kg. approximately  $\frac{1}{3}$  of the injected atabrine was recovered from the lungs. It is possible that after relatively large doses in concentrated solution, the relatively insoluble atabrine base may be precipitated in the blood and be held back by the capillary beds first encountered i.e. the lungs leading to an exceptionally high concentration in this organ.

TABLE 4

*Atabrine in tissues of chickens receiving 5 mgm./kg. of atabrine dihydrochloride intravenously and sacrificed after 1, 5 and 10 daily injections*

SAMPLE	NUMBER OF DAILY INJECTIONS					Hours after last injection	
	1		5		10		
	1	5	1	1	42		
Lung	11	12	22	17	33	28	5.9
Kidney	23	42	34	38	40	42	7.4
Spleen	62	43	64	70	81	85	10
Adrenal	53	84	67	75			29
Liver	51	88	82	136	120	133	10.8
Heart	8.6	11	11	10	12	14	8.6
Brain	5	4.6	4.4	13	8	8	
Muscle	2.8	2	2.8	2.7	2.4	2.2	0.8
Bile	6.2	16	30	15.6	14	20	29
Pancreas	26	20	29	23	23	23	3.8
Bone marrow	11	10	18	21	21	20	2.8
Testis	2.6	7	6.0	7.5	12	19	6.2
Blood plasma			0.23	0.41			0.01
Total atabrine in mgm./organ							
Lung		0.00	0.16	0.22	0.26	0.35	0.06
Kidney		0.32	0.31	0.40	0.34	0.44	0.06
Liver		1.40	2.81	3.40	2.91	4.09	0.30

In the chick both the highest tissue concentration and greatest total amount of atabrine were found in the liver. In this species in contrast to the rabbit relatively low concentrations are found in the lung even after repeated dosage.

While admittedly little emphasis can be placed on data obtained from random samples of bile, it is nevertheless of interest to note that the concentration of atabrine in chicken bile is in nearly all cases higher than that of rabbit bile.

The differences in the metabolism of atabrine in the chicken and rabbit serve to emphasize the fact that intensities of antimalarial activity of drugs may well differ in avian simian and human malaria because of metabolic differences in the host.

ATTEMPTS TO DEMONSTRATE IN VITRO DESTRUCTION OF ATABRINE 0.1 mgm samples of atabrine in 10 cc of Ringer-Locke were incubated for 6 hours with Ringer-Locke extracts of chicken and rabbit liver, lung and kidney according to a previously described technique (7). The extracts were then analysed for atabrine. No evidence for any *in vitro* destruction of the drug could be demonstrated by this procedure.

PLACENTAL TRANSMISSION OF ATABRINE Two rabbits at the 30th day of pregnancy were injected with 5 mgm /kgm atabrine intravenously, and after one hour, fetal and maternal tissues were analysed for atabrine. One animal

TABLE 5  
*Atabrine in maternal and fetal tissues*

Rabbits I (wt 3.4 kg) and II (wt 5.7 kg) received a single intravenous dose of 5 mgm /kg and were sacrificed one hour later. Ia represents tissues from a single fetus born 35 minutes after the atabrine injection. Ib represents pooled samples from two unborn fetuses. Rabbit III received 3 daily intravenous injections of 5 mgm/kg of atabrine. The fetus was delivered and analysed approximately 24 hours after the third injection.

SAMPLE	RABBIT NUMBER			mgm /kg
	I	II	III	
<i>Maternal organs</i>				
	mgm /kg	mgm /kg	mgm /organ	mgm /kg
Lung	23	293	4.54	
Kidney	22	98	1.91	
Spleen		2		
Liver	21	6.8	1.08	
Heart		47		
Placenta	8.2	2.0		
<i>Fetal organs</i>				
	a	b		
Lungs	2.9	3.5	1.0	10.0
Liver	3.3	3.0	4.4	6.3
Kidney	trace	1.5	trace	5.5
Brain				1.5
Placenta		27.5	20.0	

started to deliver 30 minutes after injection. Fetal analyses in this case were done on the fetus born 35 minutes after the injection, and two which were undelivered at the end of the hour. In the second rabbit, analyses were done on pooled samples from 5 fetuses. This rabbit had severe convulsions following the injection of atabrine and remained in a very depressed condition until sacrificed. The fetuses were alive and appeared normal. A third rabbit received three daily intravenous injections of 5 mgm /kgm of atabrine. This animal delivered one living fetus approximately 24 hours after the last injection of atabrine.

The data from these three experiments are presented in table 5. Appreable amounts of atabrine are present in fetal lungs, kidney and liver after ad ministration of atabrine to the mother. Repeated doses to the mother evidently result in increased storage in the fetuses since the offspring of rabbit III showed considerably higher concentration of atabrine than those of the other two although analyses were done 24 hours after the final dose. A considerably higher concentration of atabrine is withheld by the fetal than by the maternal placenta.

These experiments show that atabrine injected into the maternal circulation passes through the placenta and accumulates in the fetal organs. While we are not aware of any evidence that atabrine has proved toxic to the human fetus nevertheless our experiments suggest that if the findings in the rabbit obtain in the human large or repeated doses might be harmful to the fetus.

#### SUMMARY

The accumulation and persistence of atabrine in the tissues of the fowl and rabbit has been studied after single and repeated intravenous injections.

Atabrine was found to be more rapidly eliminated in the fowl than in the rabbit.

In the rabbit atabrine is stored in highest concentration in the lung, kidney, spleen, adrenal and liver. However when organ weights are taken into consideration, the greatest amount of atabrine is generally stored in the liver. In the chicken little atabrine is stored in the lungs and with the exception of the liver all organs store less atabrine than do those of the rabbit.

The intravenous toxicity of atabrine is approximately three times greater in the rabbit than in the fowl.

Atabrine traverses the placenta of the pregnant rabbit and becomes stored in the fetal tissues.

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## STUDIES ON PAIN

### THE EFFECTS OF ANALGESIC AGENTS ON SENSATIONS OTHER THAN PAIN

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The effects of analgesics of the opiate group on sensations other than pain have received relatively little attention. Eddy (1) has reviewed exhaustively the literature on the effects of morphine on hearing, smell and cutaneous sensations, and the reported observations are inconclusive and contradictory. Study of the effects of analgesic agents such as acetylsalicylic acid, the barbiturates and ethyl alcohol on sensations other than pain has been almost entirely neglected.

It was the purpose of this study to compare in man effects of various analgesic agents on the pain threshold with effects on the sensations of hearing, smell, touch, two-point discrimination and vibration. It has already been established in recent quantitative studies (2, 3 and 4) that the pain threshold in man can be elevated as much as 80 to 100 per cent by morphine sulfate (0.015 to 0.030 gm.), 60 per cent by codeine phosphate (0.60 gm.), 50 per cent by ethyl alcohol (30 to 60 cc. 95%), 35 per cent by acetylsalicylic acid (0.3 to 1.8 gm.), and a minimal amount by the barbiturates.

The subjects for these experiments were the three authors and three additional volunteers. The subjects included five men and one woman, in an age group from 23 to 45 years and a weight group from 68 to 80 kilograms. All were healthy, interested and well-trained observers.

**METHODS** Thresholds for hearing were measured with a 6B Western Electric Audiometer at frequencies of 128, 512, 2048 and 9747 cycles per second in a soundproof room. Threshold for smell were measured with an Elsberg Quantitative Smell Function apparatus, with two odors, coffee and citral.

Tests for touch perception and two-point discrimination were made within an area 10 by 10 cm. on the inner aspect of the arm 2 to 4 cm. below the antecubital fossa. The area was cleanly shaven and outlined in ink. Care was taken not to touch the same spot twice in succession, and to include systematically the entire 100 cm.<sup>2</sup> area in testing. For the assay of touch sensation von Frey hairs were used which at a given length gave rise to a sensation of touch, but which failed to give such a sensation when the length was increased.

A metal divider with blunt points was used for two-point discrimination. Care was taken to touch the skin with both points simultaneously and horizontally to the longitudinal axis of the arm, and with uniform pressure each time. The "end point" for the threshold of two-point discrimination was taken

as the distance between two points touched on the skin which could be correctly recognized as two points in four out of five trials.

The threshold for vibration sense was measured with a Roth Neurometer. This instrument is a tuning fork with a cross piece by which it can be suspended from the index and middle fingers between the first and second joints. The time is recorded by a stop watch from the moment of striking the tuning fork until vibration is no longer perceived.<sup>1</sup>

**PROCEDURE.** Experiments were begun 2 to 3 hours after breakfast. It was found to be unwieldy to have more than 4 subjects participating in any one experiment. Subjects A, W and H, G kept records on each other and on the other participating subjects. All records of data were kept secret from the subjects until the end of the experiment. Two sets of control observations were

TABLE 1

SENSATION	AVERAGE CONTROL READING	RANGE
Two point discrimination	3.7 cm (4 correct out of 5 trials)	2.3-5.1 cm.
Touch	von Frey hair 8.0 cm in length	5.0-10.0 cm
Vibration sense	25 seconds	33-37 seconds
Smell	5 cc (citral & coffee)	3 cc to 15 cc
Hearing frequencies 128	+2 (dial units)	-6 to +10
512	-3 (dial units)	-11 to +5
2048	-2 (dial units)	-12 to +8
9747	-11 (dial units)	-18 to -4

The wide range in table 1 suggests a considerable fluctuation. This is however more apparent than real since any single individual did not vary appreciably. Thus with 2 point discrimination the range was  $\pm 20\%$  individually with touch, vibration sense, smell and hearing it was not more than  $\pm 5\%$ . Since all comparisons were made in terms of a given individual on a given day before and after analgesia, differences in threshold from one individual to another as indicated in table 1 had no significance.

made at about 30 minute intervals. Then the analgesic agent was administered and, during a period of 2 to 7 hours afterward readings of the various thresholds were made at 15 to 30 minute intervals. Twenty-seven series of observations were made during a period of 8 weeks. In 17 of these the subjects knew the nature of the analgesic administered. In 5 the nature of the substance was unknown to the subjects. In addition 5 placebos were administered.

**RESULTS** Control threshold readings for the various sensations were estab-

<sup>1</sup> An attempt was made to include studies on weight discrimination. Black cubes (0.875 in<sup>3</sup>) weighing 3, 6, 9, 12 and 15 gm. were utilized. The subject was required to arrange them in order of increasing weight with the eyes closed. The time required for the performances varied from 11 to 39 seconds in the six subjects. Following administration of the analgesic agents, the variation in the time for successful arrangement of the blocks remained approximately the same.

lished for the individual subjects at the beginning of each series of observations before administration of the analgesic agent. The averages of 40 such control readings for each sensation studied in all of the six subjects are as follows:

In fig. 1 are shown the observations on the thresholds for touch, hearing, smell and vibration sense and on two point discrimination before and after administration of 0.015 gm. morphine sulfate. Each point charted represents the average of 7 series of observations on 4 subjects. In contrast to the lack of effect on the non-painful sensations there is shown the pain threshold raising effect of a

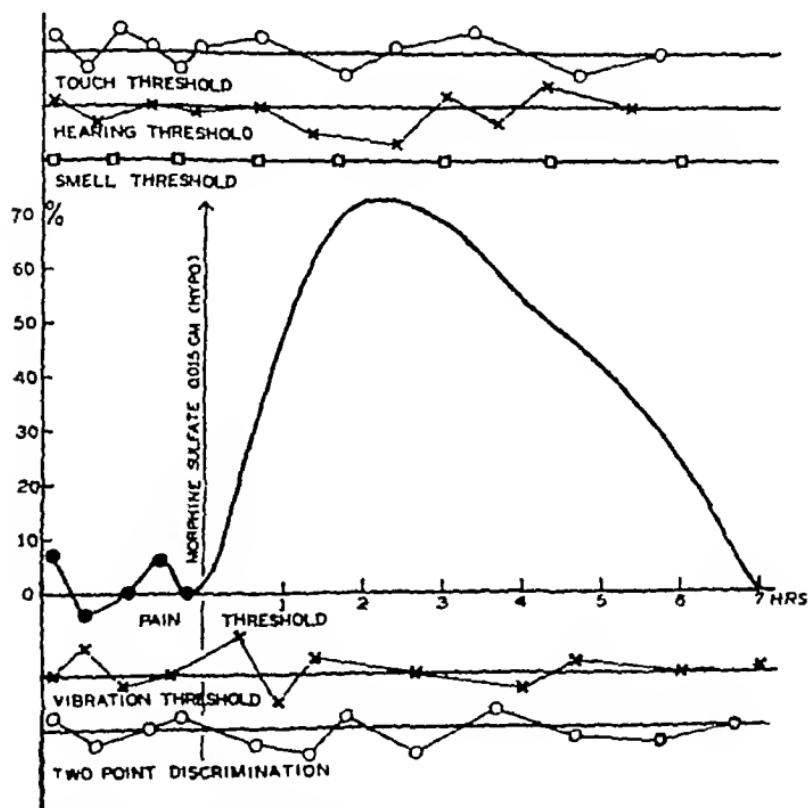


FIG. 1. The effect of morphine sulfate on the thresholds for touch (von Frey hairs), hearing (audiometer), smell (Elsberg apparatus), vibration (Roth neurometer), and on two point discrimination—compared with its effect on the pain threshold.

like amount of morphine sulfate reported by observers using the Hardy apparatus (5).

The effects of ethyl alcohol, 60 c. 95% in 200 cc of iced sweetened water ingested within three minutes are shown in fig. 2. Here again it is seen that ethyl alcohol, though altering the ability to perceive noxious cutaneous stimuli, as shown by the curve of the effects on the pain threshold, has little if any significant effect on the threshold of perception of the sensations of smell, hearing, vibration, touch and two-point discrimination.

A similar lack of effect on the thresholds of perception of touch, hearing, smell, and vibration sense was observed after oral administration of 0.3, 0.6 and 1.8 gm. acetylsalicylic acid, 0.5 gm. of the barbiturate Evipal (N methylcyclohexenylmethyl barbituric acid) and the subcutaneous injection of codeine phos-

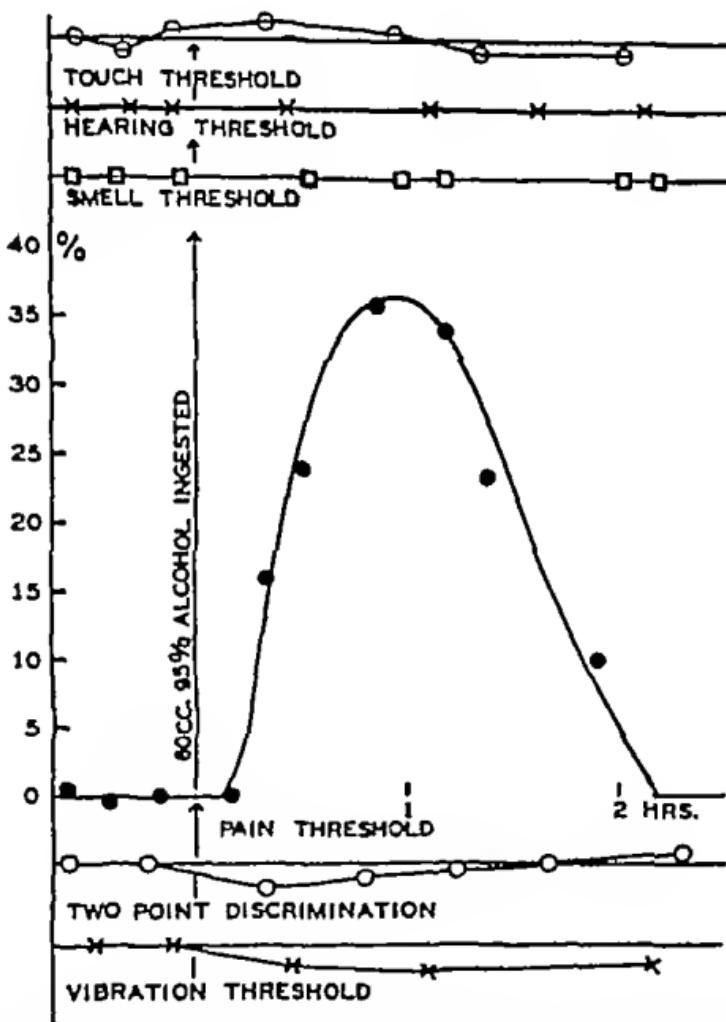


FIG 2. The effect of ethyl alcohol (80 cc 95%) on the thresholds for touch (von Frey hairs), hearing (audiometer), smell (Elaberg apparatus), vibration (Roth neurometer) and on two-point discrimination—compared with its effect on the pain threshold

phate 0.60 gm. Two-point discrimination was likewise unaltered by any of these agents except acetylsalicylic acid in amounts of 1.8 gm. which definitely raised the threshold of this sensation in two subjects. During the second hour after the ingestion of such an amount of acetylsalicylic acid, the minimal distance

between two points which could be recognized as such in 4 out of 5 trials was twice as great as in control observations made on that day

**DISCUSSION** Whereas pain thresholds vary relatively little ( $\pm$  15 per cent) in normal human subjects (6) olfactory thresholds have been reported to vary 100 per cent by the Elsberg apparatus (7) and vibratory thresholds 33 per cent on various parts of the body (8) to practically no variation at the finger tips (9). Observations in the literature on the sensations of touch and two-point discrimination have not been concerned with threshold variation. We have therefore used our own controls (Table 1) as bases for comparison for the action of the analgesics.

"Therapeutic" amounts of the analgesic agents morphine sulfate, codeine phosphate, acetylsalicylic acid, ethyl alcohol (95%) and a barbiturate ("Evipal") were observed not to alter the threshold for perception of touch, vibration, hearing, smell and two-point discrimination. The data here reported on auditory thresholds are in agreement with observations of Andrews (10) who found that morphine had no effect on thresholds of perception of hearing tones of 200, 1000 and 5000 cycles per second.

These experiments, furthermore, indicate that the specificity of the pain threshold raising action of morphine is not unique, since the other agents studied likewise had no effect on the thresholds of sensations other than pain. The fact that a wide variety of agents are able to alter the pain threshold selectively provides further evidence that pain is a specific sensory experience with its own neural structures and properties.

#### SUMMARY

The thresholds of perception of sensations other than pain—touch, vibration, two-point discrimination, smell and hearing—were not raised by "therapeutic" amounts of morphine sulfate, codeine phosphate, ethyl alcohol (95%), a barbiturate ("Evipal") and acetylsalicylic acid.

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